BRAINSTEM PAIN-MODULATING NEURONS “SEE THE LIGHT”

By

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CERTIFICATE OF APPROVAL

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LIST OF ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CeA	Central nucleus of the amygdala
CFA	Complete Freund's adjuvant
CPM	Conditioned pain modulation
DMH	Dorsal medial nucleus of the hypothalamus
DNIC	Descending noxious inhibitory control
GABA	γ-Aminobutyric acid
H	Heat
ipRGC	Intrinsically photosensitivity retinal ganglion cell
NMDA	N-methyl-D-aspartate
OPt	Olivary pretectal nucleus
PAG	Periaqueductal gray
PB	Parabrachial nucleus
PO	Posterior thalamic nucleus
PW	Paw withdrawal
RVM	Rostral ventromedial medulla
TMD	Temporomandibular joint disorder

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ABSTRACT

Functional pain disorders are highly prevalent, but difficult to diagnose and treat. There is strong evidence that dysfunction in central pain modulation contributes to pain amplification and hypersensitivity. An important system in normal and pathological pain is a brainstem pain-modulation circuit that regulates nociceptive transmission through direct projections to the dorsal horn and trigeminal nucleus. The rostral ventromedial medulla (RVM) is the output of this system and facilitates or inhibits pain through two classes of cells termed “ON-cells” and “OFF-cells”, respectively. These cells are defined physiologically based on changes in firing during response to noxious stimulation; ON-cells are activated and have a “burst” in activity, while OFF-cells are inhibited and have a “pause” in ongoing activity. While the role of RVM in nociception has been well-characterized in male animals, very little is known about RVM activity in females. However, chronic pain disorders are more prevalent in females, and animal research has revealed sexual dimorphisms in other parts of this pain-modulation system. Therefore, it is important to characterize RVM activity in females, as sex differences could have clinically relevant implications. Thus, the first aim of this thesis was to compare the basic firing properties and light-evoked stimulus-response curves of RVM cells in female versus male rats.

RVM response to light is of interest because a subset of RVM neurons in males respond to visual light, and while many chronic pain patients report abnormal sensitivity to light, the neural pathways contributing to light-evoked discomfort are unknown. Light-evoked activation of pro-nociceptive ON-cells and suppression of anti-nociceptive OFF-cells could provide a means for ambient light to modulate pain transmission. Additionally, RVM is plastic and develops enhanced responses to somatic stimuli following injury. If RVM response to light is similarly enhanced during injury, RVM could contribute to light-

evoked discomfort. Therefore, the second aim of this thesis was to determine if RVM cell light-evoked stimulus-response curves are altered during persistent inflammation.

I found that under basal conditions and in persistent inflammation, the responses of RVM cells to noxious heat and visual light are similar in the two sexes, showing that the overall output of the descending pain-modulation system is comparable. Further, RVM cells respond to very low levels of light and the response to light is graded with stimulus intensity, which is in contrast to somatic stimuli-evoked all-or-nothing responses. These data argue for differences in how light and somatic stimuli engage RVM, and suggest that light-related information represents a “top-down” input to RVM. In addition, inflammation shifted the stimulus-response curve of pain-inhibiting OFF-cells, such that they are inactivated at a lower threshold and to a greater degree by dim levels of light. Therefore, in pain states, decreased descending inhibition of pain during exposure to light could contribute to photophobia.

In summary, these findings demonstrate that despite sexual dimorphisms in pain-modulation circuitry at the molecular level, the output of the system is comparable. This work also provides a greater understanding of the interaction between pain-modulation systems and visual light, and contributes to our understanding of photophobia.

CHAPTER 1

INTRODUCTION

1.1 Overview

Pain is an important and protective sensory experience that alerts us to actual or potential tissue injury. Noxious or potentially harmful stimuli activate nociceptors in the periphery that transmit this information to the central nervous system where it is perceived as pain. The transmission of nociceptive information is highly regulated, and can be enhanced or dampened depending on biological needs and behavioral priorities. Nociception is inhibited during times of extreme emotional stress, allowing an organism to prioritize survival over minor injuries. For example, during escape from a predator, “stress-induced analgesia” leads to less pain being perceived from a given injury. On the other hand, nociception can be enhanced, acting in a protective manner to prevent further tissue damage, as with illness or while healing from a physical injury. However, this state of enhanced pain, or hyperalgesia, can become pathological, with increased pain perception occurring even in the absence of injury or after injury has resolved, as in chronic pain disorders. This bidirectional control over pain transmission is largely influenced by descending pain-modulation circuitry in the brainstem, and it is likely that there is maladaptive-plasticity in this circuitry that contributes to persistent pain.

Along with hypersensitivity to somatic stimulation, many patients with functional pain disorders experience multi-sensory hypersensitivity, including increased sensitivity to light, a symptom termed photophobia. These patients report that normal levels of light are uncomfortable, and that light can exacerbate or induce pain. While we know somatosensory hypersensitivity is the result of dysfunction in multiple levels of the complex neural network that regulates pain, there is much less known about the interactions of light with pain-modulation circuitry.

One way in which light could induce pain is through the rostral ventromedial medulla (RVM), the output node of a major pain-modulation system. The RVM is a brainstem

structure that exerts bidirectional control over nociception through direct, descending projections to the dorsal horn and trigeminal nucleus. It receives input from higher structures that are responsible for emotion and cognition, and thus is an important site of integration. RVM is also a significant contributor to both normal and pathological pain. Importantly, RVM is plastic and develops enhanced responses to somatic stimuli during injury, mediating hypersensitivity. There is evidence that light interacts with pain-modulating neurons in RVM. Thus if RVM response to light is similarly altered during injury, RVM could contribute to photophobia in chronic pain conditions.

The goal of this thesis was to characterize RVM cell responses to visual light by establishing stimulus-response curves, and to determine if responses to light are altered during persistent inflammation. A major gap in our understanding of pain-modulation systems was also addressed by performing the experiments in both male and female animals to determine if there are sex-related differences in RVM activity. This latter point is important because chronic pain disorders disproportionately impact females, and we know there are other sexual dimorphisms in pain-modulation circuitry, but the vast majority of work characterizing RVM has been done in male animals. Here, I use *in vivo* electrophysiology to establish stimulus-response curves for RVM cell response to light, and validate the use of female animals in RVM recording studies. In summary, I showed that RVM responds to light in a graded manner, and that the responses are similar in males and females. Further, I showed that in animals with persistent inflammation, compared to in control animals, activity of RVM pain-inhibiting neurons is significantly more depressed by dim light.

1.2 Modulation of nociception by the rostral ventromedial medulla

1.2.1 Anatomy and connectivity of a pain-modulation system

Functionally, the RVM is defined as the midline pontomedullary area in which electrical stimulation or opioid microinjection produces behavioral antinociception (Zorman et al., 1981). However, experimental activation in the absence of noxious stimuli does not produce changes in behavior or sensation showing the RVM is specific to pain (Mayer et al., 1971, Waters et al., 1997). Structurally, it includes the raphe magnus and adjacent reticular formation ventral to the gigantocellular reticular nucleus and medial to the facial nucleus (Fields et al., 1999). RVM exerts descending control through direct projections to both deep and superficial dorsal horn and to areas of the trigeminal nucleus that are important to nociception (Basbaum et al., 1978, Fritschy et al., 1987, Ruda et al., 1981, Fields et al., 1995, Sugiyo et al., 2005). An individual RVM neuron can have diffuse projections, terminating in multiple levels of the dorsal horn, as well as the trigeminal nucleus (Lovick et al., 1983, Huisman et al., 1981, Basbaum et al., 1976). Thus, the RVM is the final output of a complex, interconnected network for pain modulation, that can both inhibit pain, such as during opioid or stress-induced analgesia, and facilitate pain, such as while healing from an injury.

Most ascending information about noxious stimuli is transmitted to RVM indirectly, but there are also a few direct projections from the dorsal horn and trigeminal nucleus (Sugiyo et al., 2005, Wang et al., 1999). Nociceptive signals are first transmitted to the central nervous system by peripheral C- and Aδ-fibers that synapse in either the spinal or trigeminal dorsal horn. Dorsal horn neurons then project to higher structures in the brain including parabrachial nucleus (PB) and periaqueductal gray (PAG), that are critical relays to RVM (Abols et al., 1981, Beitz et al., 1983, Beitz, 1982a, Roeder et al., 2016, Keay et al., 1997).

The RVM is also the final convergence site for many “top-down” influences (Calejesan et al., 2000, McGaraughty et al., 2002, Wagner et al., 2013, Sandkühler et

al., 1984, Basbaum et al., 1984). Projections from higher structures are relayed to RVM through the PAG, which integrates inputs from the amygdala, medial prefrontal cortical areas (e.g. anterior cingulate), hypothalamus, and preoptic areas (Figure 1) (Mantyh, 1982b, Aggleton, 1993, Beitz, 1982b, Floyd et al., 2000, Rizvi et al., 1991). These brain regions play important roles in emotion and cognition. Functionally, stimulation of PAG inhibits nociceptive transmission, but PAG itself has very few projections to the dorsal horn, and antinociception is dependent on its connection to RVM (Basbaum et al., 1979, Basbaum et al., 1976). However, RVM is not a simple relay as it is reciprocally connected to PAG (Mantyh, 1982a, Fields et al., 1985). Therefore, RVM is the final output of converging descending and ascending pathways, forming a feedback loop that allows emotional and cognitive factors to influence nociceptive transmission. This circuit is an important contributor to both normal and pathological pain (Heinricher et al., 2009, Heinricher et al., 2013, Porreca et al., 2001, Ren et al., 2002).

1.2.2 RVM exerts bidirectional control through ON- and OFF-cells

The RVM exerts bidirectional control over nociception through two classes of cells, termed ON- and OFF-cells, that project directly to the spinal and trigeminal dorsal horns. These cells were named based on physiological responses; activity of ON-cells increases (or “bursts”), whereas activity of OFF-cells ceases (or “pauses”) just prior to behavioral responses to noxious stimuli (Figure 2) (Fields et al., 1983a). These two cell classes, respectively amplify and suppress nociceptive transmission, producing a net hyperalgesia or hypoalgesia (Fields, 1992, Porreca et al., 2002, Heinricher et al., 2009, Zhuo et al., 1992). Other neurons in RVM are referred to as “NEUTRAL-cells,” and whether they have a role in pain modulation is unknown but seems unlikely (Fields et al., 1985). Extensive work has gone into characterizing the physiology and function of these

cells in male animals, but only a few studies have been performed in female animals (see section 1.4.2).

ON-cells have a “burst” in activity in response to noxious stimuli that can be quantified by the total number of action potentials, duration of burst, or peak firing rate. ON-cells exert a pronociceptive effect (Barbaro et al., 1986, Fields et al., 1988), and manipulations that selectively and directly increase ON-cell firing cause hyperalgesia (Neubert et al., 2004). Additionally, ON-cells express μ-opioid receptors and thus respond to opioids administered directly in the RVM (Heinricher et al., 1992, Heinricher et al., 1994). While morphine acts directly on ON-cells to suppress activity, contributing to analgesia, ON-cell suppression alone is not sufficient to produce analgesia, but may be important to the actions of opioids during inflammation (Heinricher et al., 1999, Heinricher et al., 2009, Porreca et al., 2002, Heinricher et al., 1994).

In contrast, OFF-cell ongoing firing ceases, or “pauses” during reflexive-withdrawal from noxious stimuli, and responses can be quantified by the duration of time that the cell is quiet or as percent suppression during the nociceptive reflex. This “pause” is required for behavioral response to noxious stimuli (Fields et al., 1983a, Heinricher et al., 2010a). OFF-cells exert an antinociceptive effect through descending inhibition at the dorsal horn, and manipulations that eliminate the OFF-cell pause produce analgesia (Fields et al., 1985, Heinricher et al., 2010b, Heinricher et al., 2010a). For example, opioids and non-opioid analgesics, such as morphine or improgan, indirectly act on OFF-cells to increase firing rates and abolish the reflex-related pause, resulting in analgesia (Heinricher et al., 1994, Fields et al., 1983b, Heinricher et al., 2010b, Heinricher et al., 2010a). Further, the pause in OFF-cell activity is necessary to the actions of analgesics.

Another characteristic of RVM cells is that reflex-related firing changes occur just prior to nociceptive withdrawal and are also observed in paralyzed animals (Fields et al.,

1985). Thus the reflex-related responses are not secondary to motor responses. Additionally, within each class of cells, firing is generally in phase, meaning that during times of high activity within one class most cells of that given class exhibit high activity, and vice versa (Barbaro et al., 1989, Heinricher et al., 1989). Between the two cell classes, ON- and OFF-cell ongoing and reflex-related activity is out of phase, meaning that during an OFF-cell active phase, ON-cells are generally inactive, and vice versa. These similarities and differences reflect that while ON- and OFF-cells act in concert to modulate nociception, they are distinct groups of cells, and allow RVM to exert net inhibitory and facilitatory effects on nociception.

Finally, the third class of RVM cells, termed NEUTRAL-cells, does not have an apparent or yet identified role in pain modulation, and is physiologically and pharmacologically distinct from ON- and OFF-cells. NEUTRAL-cell firing does not change during withdrawal to nociceptive stimulation nor during administration of opioids or other manipulations that affect ON- and OFF-cell firing (Heinricher et al., 1992, Harasawa et al., 2000, Winkler et al., 2006, Xu et al., 2007). Alternatively it has been suggested that NEUTRAL-cells may develop ON- and OFF-cell properties during inflammation (Miki et al., 2002), but baseline activity prior to inflammation was not recorded in that study, so it is possible cells were mischaracterized. Moreover, others report no change in NEUTRAL-cells during inflammation (Cleary et al., 2013, Kincaid et al., 2006). However, a subset of NEUTRAL-cells are serotonergic, and serotonin likely contributes to nociceptive modulation, so there could be some unidentified role of NEUTRAL-cells in pain modulation (Mason, 1997, Potrebic et al., 1994).

1.2.3 Plasticity of RVM in acute and persistent pain

There are distinct physiological and biochemical changes in brainstem pain-modulation circuitry during acute and persistent pain. While RVM is plastic and cell

activity is altered during inflammation and injury, the changes depend on the duration of injury and vary as injury moves from an acute state to a persistent state. During acute injury, there are changes in the ongoing activity of RVM cells that have a net pain facilitatory effect and contribute to hypersensitivity. Meanwhile, in persistent injury, ongoing activity returns to baseline, and RVM has a net pain inhibitory effect, although cells develop more robust responses to noxious stimuli. There are also distinct molecular changes that take place as pain persists (Pinto et al., 2007). For example, as inflammation progresses, the efficacy of opioids injected into the RVM increases (Hurley et al., 2000, Schepers et al., 2008). There are also changes in excitatory amino acid neurotransmission. For example, NMDA administered in the RVM has faciliatory effects on hyperalgesia during the first few hours, but inhibitory effects at later timepoints (Terayama et al., 2000, Guan et al., 2002, Xu et al., 2007). While, AMPA administration inhibits nociception at early and later timepoints, inhibition is greater at later timepoints (Guan et al., 2002, Guan et al., 2003). Thus while acute and chronic injuries may present with similar symptoms, the role and behavior of pain-modulation circuitry under each condition is distinct.

The ongoing activity at the onset of stimulation sets the nociceptive withdrawal threshold, and a shift in the balance between pain facilitation and pain inhibition mediates hyperalgesia (Heinricher et al., 1989, Heinricher et al., 1991, Heinricher et al., 1999). During early phases of injury, the ongoing activity of RVM cells is shifted to a pronociceptive state, meaning there is an increase in ON-cell and decrease in OFF-cell activity, leading to increased responsiveness of dorsal horn nociceptive neurons (Cleary et al., 2013, Carlson et al., 2007). Changes in ongoing firing are associated with hyperalgesia during acute injection of Complete Freund's Adjuvant (CFA), topical application of mustard oil, dural inflammation, or opioid withdrawal. Moreover, in these

models of acute pain, silencing or blocking RVM attenuates hyperalgesia and allodynia (Cleary et al., 2013, Kincaid et al., 2006, Edelmayer et al., 2009, Bederson et al., 1990, Kaplan et al., 1991). Additionally, blocking NMDA receptors in RVM attenuates mustard oil-induced hyperalgesia (Xu et al., 2007). This is most likely mediated by ON-cells, because the increased ON-cell activity associated with mustard oil application is reversed by NMDA antagonist, but there is no effect on OFF-cells. These findings show clear effects of acute inflammation or injury on the nociceptive “tone” of RVM, reflecting the importance of ON-cell drive and the influence of descending facilitation in acute hypersensitivity.

By contrast, during prolonged inflammation or injury, ongoing activity returns to baseline, but reflex-evoked responses of RVM cells are enhanced, and prolonged OFF-cell pause and ON-cell burst are observed (Cleary et al., 2013, Carlson et al., 2007). The duration of pause or burst likely corresponds to a state of increased vigilance, because during this time ON- and OFF-cells are in a net pro-nociceptive state (Heinricher et al., 1989), so a subsequent stimulus can evoke a response at a lower threshold (Ramirez et al., 1989). Furthermore, ON- and OFF-cells are “sensitized” to stimulation and respond to mechanical stimuli normally considered innocuous (Cleary et al., 2013, Carlson et al., 2007, Gonçalves et al., 2007). While, lowered cell response thresholds may help maintain behavioral hypersensitivity, the influence of RVM on pain is complex, and in contrast to acute injury, net descending inhibitory output from RVM prevails during prolonged inflammation. Blocking RVM in animals with persistent inflammation enhances behavioral hypersensitivity (Cleary et al., 2013, Gonçalves et al., 2007, Wei et al., 1999) and increases the sensitivity and receptive fields of dorsal horn neurons (Ren et al., 1996). This demonstrates that RVM develops a compensatory response to persistent inflammation and net descending inhibitory output predominates.

Taken as a whole, these findings demonstrate that the net RVM output is plastic, and that the balance between pain facilitation and inhibition fluctuates with time as pain persists.

1.3 Sex differences in pain experience

Prior to the 1990's sex as a biological variable was largely ignored, and almost half of published articles in basic neuroscience research did not even report the sex of the animal model employed (Berkley, 1992). However, simply extending findings from males to females, or ignoring sex entirely, can result in missing important biological underpinnings that may lead to sex-related differences. An extreme example is that in the 1990's the FDA had to withdraw multiple prescription drugs, primarily because there were unexpected side effects in women that were missed due to a lack of female subjects in preclinical studies (Lee, 2018). While interest in sex differences increased during the 1990's and 2000's, there remain many unknowns, and most biomedical studies are still performed exclusively in males (Beery et al., 2011). Thus, in the mid 2010's, the NIH, along with other funding agencies, developed policies regarding integration of sex as a biological variable in biomedical research (Clayton et al., 2014). This initiative has not been without criticism based on concerns that adding female animals will lead to more costly experiments and slow the pace of biomedical research, due to the need for increased sample size or potentially increased variability and decreased power (Fields, 2014, Eliot et al., 2016). However, if implemented properly, consideration of sex as a biological variable will better inform our understanding of disease mechanisms.

Within the pain field, the problem of not considering sex was made clear in the late 1990's when several reviews pointed out that clinical pain disorders disproportionately impact females, and that there may be sex differences in pain sensitivity in healthy

individuals (Fillingim et al., 1995, Unruh, 1996, Riley et al., 1998). While failure to report sex of subjects in neuroscience articles has decreased, a lack of consideration for sex as a variable is still highly prevalent. For example, approximately 80% of studies published in *Pain* between 1996 and 2005 were conducted in males only (Mogil et al., 2005, Will et al., 2017). Much of the pain-related research into sex differences during the past 20 years has focused on identifying if there are differences in pain sensitivity between males and females, because higher pain sensitivity in females could contribute to the sex-related disparities in chronic pain. On the whole, results are highly variable and often conflicting. While many reviews or meta-analyses have attempted to reconcile and explain the conflicting results, and overall it is generally acknowledged that at least some sex-related differences in pain exist, there is not a lot of agreement on exactly what differences exist or to what degree (Fillingim et al., 2009, Racine et al., 2012a, Aloisi, 2003, Craft et al., 2004a, Traub et al., 2013, Mogil, 2018, Greenspan et al., 2007, Greenspan et al., 2013).

In the following subsections, clinical and experimental human data, as well as animal research pertaining to differences in pain sensitivity and experience will be summarized. While, chronic pain disorders have a greater prevalence in females, sex-related differences in reported pain severity from clinical patients and findings on experimentally-induced pain response in both humans and animals are of lesser magnitude. Potential differences are often complicated and variable, if present at all.

1.3.1 Clinical pain

Based on data from clinical populations, most pain disorders disproportionately impact females (Greenspan et al., 2013). However, these findings are potentially skewed by biopsychosocial factors as women are more willing to report pain (Isacson et al., 2002) and more likely to utilize healthcare systems (Fillingim et al., 2009, Mogil, 2012).

The framework through which diagnoses are made also impacts the chances of diagnosing chronic pain in males compared to females (Jones et al., 2015). Therefore, the fact that more patients in pain clinics are female, is not necessarily representative of the population as a whole and is likely a biased sample. Population-based studies generally affirm the greater prevalence of chronic pain in females, although these studies often find that the disparity is less than what would be predicted by clinical populations (Greenspan et al., 2013, Fillingim et al., 2009, Bartley et al., 2013, Sarlani et al., 2005, Slade et al., 2011).

For example, the female-to-male prevalence of temporomandibular joint disorder (TMD) within clinical populations is approximately 9:1 but within the general public it is approximately 2:1 (LeResche, 2000). The Orofacial Pain Prospective Evaluation and Risk Assessment Study (OPPERA Study), one of the largest population-based case-control studies to date, examined biological, psychosocial, and genetic risk factors that are associated with the onset of TMD in healthy controls and with cases of chronic TMD. Interestingly, within the healthy control groups, there was a trend towards a higher prevalence of first onset TMD and development of preclinical signs in females, but no significant sex differences (Slade et al., 2013b, Slade et al., 2013a, Ohrbach et al., 2013, Bair et al., 2013). However, there was a significant sex difference in the prevalence of TMD within the chronic TMD case-control group (Slade et al., 2011). This finding in agreement with a large-scale US National Health Interview Survey that found the prevalence of chronic TMD within the general population is higher in females than males (Isong et al., 2008). The findings from the OPPERA Study reflect that sex may not be a predictor of the prevalence of preclinical signs and acute symptoms, but that sex may have an association with the likelihood of acute pain becoming persistent. However, more work and long-term studies will be needed in TMD cases and other chronic pain

disorders to determine if acute pain is more likely to become chronic in females than males.

While pain disorders impact a greater percent of females, sex differences in the severity of reported symptoms associated with a given disorder are less clear (Fillingim et al., 2009, Racine et al., 2012a). Females present with more severe pain complaints in many disorders (Unruh, 1996, Lipton et al., 2001b, Mayer et al., 2004). However, differences are likely dependent on the pain condition and influenced by test procedures and symptoms examined, as males and females often present differently and describe pain differently (Greenspan et al., 2013, Jones et al., 2015). Additionally, sociocultural sex differences and gender roles also influence reported pain severity. For example, while women are more likely to seek out social support as a positive coping mechanism, they are also more likely to catastrophize and overestimate the negative consequences of pain, leading to more psychological distress (Keefe et al., 2000, Sullivan et al., 2000, Bedard et al., 1997, Jensen et al., 1994). Catastrophizing has a strong correlation in multiple musculoskeletal conditions with reported pain severity, muscle tenderness, pain-related disability, poorer treatment outcomes, and an overall decrease in quality of life (Edwards et al., 2006, Holroyd et al., 2007, Severeijns et al., 2001). Therefore, it is likely that sex differences in catastrophizing mediate some of the observed sex differences in the presentation of chronic pain disorders. In fact, it has been reported that once catastrophizing is entered into the analysis, there is no longer a significant effect of sex on pain behavior and physical disability in osteoarthritis patients (Keefe et al., 2000). Thus while chronic pain disorders are more prevalent in females, there are psychological factors that influence the clinical presentation.

1.3.2 Acute and experimental pain in humans

As mentioned previously, sex differences in acute pain sensitivity are of interest because greater sensitivity in females is a possible explanation for sex differences in chronic pain prevalence. The hypothesis is that if females are more sensitive to pain than males under baseline conditions, they will be more greatly affected by painful events, and consequently predisposed to develop chronic pain. One method to study acute pain is through comparisons of postoperative pain severity, which is also a positive risk factor for future chronic pain. However, no differences or differences in either direction have been reported, and studies are confounded by many factors including postoperative pain management, anxiety levels, and surgery type (Fillingim et al., 2009, Ip et al., 2009).

Tests using experimentally induced pain in a laboratory or clinical setting are a more controlled method to examine baseline differences in nociceptive sensitivity. Approximately half of experiments find sex differences, and when differences are observed, females are always more sensitive than males (Greenspan et al., 2013, Mogil et al., 2010). Results vary between different stimulus modalities (thermal, pressure, electrical, ischemic) or dependent variables measured (threshold, tolerance, pain rating), but even within a given nociceptive assay there are a lot of conflicting results (Lautenbacher et al., 1993, Fillingim et al., 2009, Riley et al., 1998, Racine et al., 2012a, Ohrbach et al., 2011). Moreover, when differences are found they are fairly minor, and there is a large amount of overlap in the range of pain sensitivity between males and females in the population as a whole (Ostrom et al., 2017, Greenspan et al., 2011, Berkley et al., 2006, Racine et al., 2012a, Berkley, 1995). Although it is possible that studies observing a trend towards females being more sensitive do not reach statistical significance because they are underpowered, this likely also reflects the minimal sex-

related differences in acute pain sensitivity (Mogil, 2018, Fillingim et al., 2009, Riley et al., 1998).

Along with methodological variations, gender roles, expectations, and sociocultural factors influence the results of experimental pain testing (Sullivan et al., 2000, Defrin et al., 2009, Wise et al., 2002, Alabas et al., 2012, Mattos Feijó et al., 2018). For example, both men and women believe women have lower pain thresholds than men and manipulating gender role expectations reduces sex differences observed in experimental pain tests (Robinson et al., 2003, Robinson et al., 2001). Additionally, even the gender and appearance of the person administering the test can influence sex differences in pain tolerance (Kallai et al., 2004, Levine et al., 1991). Anxiety, fear, and catastrophizing about painful events also increase sensitivity, and is greater in females, thus more greatly skewing pain perception in females (Ohrbach et al., 2011, Meulders et al., 2012, Meints et al., 2017, Nahman-Averbuch et al., 2016).

In addition to testing responses to acute noxious stimuli, dynamic pain tests that may more closely mimic aspects of clinical pain are used to test for sex differences. Results from tests using chemical irritants that produce pain or irritation lasting several minutes to hours are generally inconclusive, and overall do not show any sex differences (Fillingim et al., 2009, Racine et al., 2012a, Baad-Hansen et al., 2005). However, tests utilizing temporal summation, where repetitive noxious stimuli are delivered, show that females may be more sensitive to mechanical summation, although results from thermal summation are less clear (Sarlani et al., 2004, Greenspan et al., 2011, Ostrom et al., 2017, Fillingim et al., 1998, Robinson et al., 2004, Staud et al., 2003, Nie et al., 2005, Sarlani et al., 2002, Lautenbacher et al., 2008). Temporal summation is enhanced in clinical pain patients, and thus thought to suggest generalized hyperexcitability of central pain processing (Sarlani et al., 2005), so differences in temporal summation might reflect

that nociceptive input is more likely to be amplified in females due to differences in central processing (Price et al., 1977). However, as with acute pain tests, anxiety about pain during temporal summation tests is greater in women, and along with gender role stereotypes, may mediate observed sex differences in temporal summation (Robinson et al., 2004).

Conditioned pain modulation (CPM) aims to test the efficacy of endogenous pain-inhibitory systems by looking at the pain-inhibitory effects of one noxious stimulus on another noxious stimulus. In chronic pain conditions CPM is reduced demonstrating that there is dysfunction in central pain-modulation circuitry (Potvin et al., 2016, Gerhardt et al., 2017, Lewis et al., 2012). Studies on sex-related differences in the efficacy of CPM in healthy controls have conflicting results, but show that females may experience less CPM, and thus might have less endogenous pain inhibition, making them more vulnerable to developing widespread pain following injury (Fillingim et al., 2009, Popescu et al., 2010, Racine et al., 2012b, Granot et al., 2008, Baad-Hansen et al., 2005, Lautenbacher et al., 2008). However, as with the pain tests described above, higher catastrophizing by females has been shown to significantly mediate the relationship between sex and CPM (Weissman-Fogel et al., 2008).

Physiological reactions to pain, including pupil dilation, muscle reflexes, and cerebral activation have also been measured in an attempt to decrease gender biases, but have equally mixed results (Fillingim et al., 2009, Greenspan et al., 2013). Further, these measures do not directly test sensitivity *per se,* and there could be sexual dimorphisms in these physiological systems. Interestingly, brain imaging studies, in which males and females reported similar sensitivity to painful stimuli, found different patterns of activation demonstrating there may be differences in central processing of pain (Linnman et al., 2012, Girard-Tremblay et al., 2014).

With such small and variable results, it is questionable as to how or if sex differences in acute and dynamic pain sensitivity relate to the significant sex disparities in clinical prevalence. However, the fact that differences are modulated by cognitive and emotional factors does point to sex differences in the pain experience when taken as a whole. Therefore, shifting our focus away from attempting to quantify minor differences in behavioral sensitivity to qualitatively examining sexual dimorphisms in pain circuitry is likely to be more beneficial to understanding sex disparities in chronic pain.

1.3.3 Experimental pain in animals

Although there are sex-related differences in prevalence of chronic pain disorders and likely differences in the pain experience in humans, most animal research on pain has been conducted in males (Mogil et al., 2005). Over the past decade there has been a push to study female animals in parallel to males, but we are still far from understanding nuanced differences in pain experience and circuitry. Acute and persistent pain testing in animals is important because it removes subject-experimenter interactions and gender-associated biases. It also allows for manipulations and controls that could not be carried out in human subjects. Surprisingly, despite the idea that animal models present a method to study “purely” biological sex differences in nociceptive mechanisms, results from animal studies are even more variable than those of human studies. The following section focuses on findings from studies in rats, but results in mouse models are similar and very little work has been done in other animals.

Similar to experimental studies in humans, stimuli used to test for acute nociceptive sensitivity in animals include electric shock, noxious heat, mechanical pressure, and ischemia. Early studies employed electric shock and generally showed that females are more sensitive than males (Mogil et al., 2000). However, response to electric shock is affected by body size and other factors, so the accuracy and meaningfulness of

differences is debatable. Tests employing hot plate, noxious heat-evoked withdrawal, mechanical stimuli, or ischemia have found no difference between males and females, or differences in either direction that are not consistently related to the nociceptive assay (Loyd et al., 2008, Wang et al., 2006, Boyer et al., 1998, Bradshaw et al., 2000, Terner et al., 2005, Bobeck et al., 2009, Tershner et al., 2000, Doyle et al., 2018, Doyle et al., 2018, Cook et al., 2006, Mogil et al., 2000, Greenspan et al., 2013).

Results regarding sex differences in responses to injury and persistent pain in animal models are also conflicting. Neuropathic pain models show there are likely no differences in hypersensitivity at the onset of injury but that there are differences in duration or at later timepoints, with females generally experiencing greater hyperalgesia (Tall et al., 2001, Coyle et al., 1995, Lacroix-Fralish et al., 2006, Joseph et al., 2003a, Joseph et al., 2003b). Testing with chemical irritants has mixed results. Irritants such as capsaicin or formalin generally show females have a greater pain response, but carrageenan has been found to induce greater hyperalgesia in males (Gaumond et al., 2002, Aloisi et al., 1994, Barrett et al., 2003, Joseph et al., 2003c, Tall et al., 2004). Additionally, sex differences in the formalin test are only observed at higher concentrations, are dependent upon the dependent variable measured, and, in some studies, observed at early phases but not late phases (Gaumond et al., 2002, Pajot et al., 2003, Aloisi et al., 1995, Aloisi et al., 1994, You et al., 2006). CFA induced inflammation is longer lasting and likely a better model of chronic pain disorders but also has variable results that do not seem to be explained by test duration or stimulus (Wang et al., 2006, Cook et al., 2005, Loyd et al., 2008, Cook et al., 2006, Craft et al., 2013). Moreover, a study on streptococcal cell wall-induced polyarthritis, an alternative method to induce persistent long-lasting inflammation, showed that there is a significant

interaction between sex and strain such that the presence and direction of sex differences in hyperalgesia was dependent upon strain (Wilder et al., 1982)

One of the simplest explanations for the inconsistencies in sex differences in nociceptive sensitivity is that hormonal fluctuations result in increased variability within female responses, so combining females from all phases of the estrous cycle obscures potential sex differences. Thus, differences in nociception may only be observed during specific phases of the estrous cycle. However, evidence to support this hypothesis is lacking. First, there is no more variability in females than in males (Mogil et al., 2005, Becker et al., 2016, Prendergast et al., 2014). Additionally, of the small number of studies to test the effects of estrous status on nociceptive sensitivity, results are unclear. Many studies find no differences in pain sensitivity across the cycle, and of studies finding differences across the cycle, there is inconsistency as to which phase of the cycle corresponds to a more “pain sensitive” state (Mogil et al., 2000). Further complicating the issue is that there are multiple ways to classify and compare estrous cycle and hormonal fluctuations may actually be the mediating factor (Mogil et al., 2000, Berglund et al., 1988). Additionally, other factors such as laboratory environment, social interaction between animals, and genetics all have been shown to affect the existence and direction of sex differences in both acute and persistent nociceptive sensitivity (Mogil, 2017, Chesler et al., 2002, Kest et al., 1999, Mogil et al., 2000, Wilder et al., 1982, Lacroix-Fralish et al., 2006). Therefore, while hormones may play some role, there are many other influencing factors that likely interact to influence pain responses.

1.3.4 Qualitative sex differences in pain circuitry are likely more important than quantitative differences in behavior

In summary, while clinical pain disorders are more prevalent in females, and experimental data from human subjects indicate that females may experience pain

differently from males, data from animals is less conclusive. Small sex differences in nociceptive sensitivity may not be what is of importance considering that there are different mechanisms at play during acute compared to persistent pain, and there are likely other factors that are more important in the development of chronic pain such as genetics, hormones, anatomy, and psychosocial factors. What is likely more important than small potential differences in nociceptive sensitivity is that both human and animal data indicate that there are sexual dimorphisms in pain circuitry and processing. Even if sexual dimorphisms have similar behavioral endpoints, a better understanding of qualitative differences in pain could have implications for future treatments and greatly aid in our understanding of chronic pain. Furthermore, sexual dimorphisms in pain circuitry mean it is critical to include female animals in studies on pain even if behavioral differences are not found.

1.4 Sex differences in pain-modulation circuitry

1.4.1 Sexual dimorphisms in pain-modulation circuitry

One line of evidence for sexual dimorphisms in pain circuitry comes from human and animal studies showing that there are sex differences in the effectiveness of opioids, which act through pain modulation systems. In humans, most studies show opioids are more effective in females than males, but, as with pain testing, there are likely psychological other factors contributing to these findings (Miller et al., 2004, Cepeda et al., 2002, Cepeda et al., 2003, Craft, 2003). Work in rats generally shows that males are more responsive to opioids than females in both acute pain tests and during persistent pain, but results vary somewhat depending on rat strain and other methodological factors such as dosing, opioid type, and pain testing assay (Doyle et al., 2018, Loyd et al., 2008, Lomas et al., 2007, Wang et al., 2006, Ji et al., 2006, Boyer et al., 1998, Cook et al., 2005, Bobeck et al., 2009, Tershner et al., 2000, Craft, 2003, Ji et al., 2006).

Although sex hormones may influence opioid-sensitivity, effects of hormone manipulations are not always observed and, when present, inconsistent (Loyd et al., 2008, Terner et al., 2005, Bernal et al., 2007, Stoffel et al., 2003, Ji et al., 2007, Mogil et al., 2000). Thus it is unlikely that hormones fully explain sex differences in opioid effectiveness. Additionally, it is unlikely that sex differences in opioid-analgesia are due to basal differences in nociceptive sensitivity, as the majority of these studies found no differences in baseline nociception. It is likely that there are sex differences in the circuitry that mediates analgesia, including PAG-RVM output, which is critical to opioid-analgesia (Loyd et al., 2009).

Both human and animal literature show that there is a similar organization in PAG-RVM connectivity in males and females (Linnman et al., 2012, Loyd et al., 2007, Loyd et al., 2006, Kong et al., 2010). However, animal work shows there are functional and molecular sexual dimorphisms in PAG-RVM circuitry. There is a greater number of PAG-RVM output neurons in female than male rats, but there is greater activation of PAG-RVM output neurons during inflammation or systemic morphine administration in males (Loyd et al., 2006, Loyd et al., 2007). Additionally, morphine significantly reduces the number of PAG-RVM neurons activated by inflammation in males but not females (Loyd et al., 2006), reflecting sex-related differences in opioid receptor expression and signaling in pain-modulation circuitry (Bernal et al., 2007, Tonsfeldt et al., 2016, Boyer et al., 1998, Tershner et al., 2000, Doyle et al., 2017, Doyle et al., 2018, Loyd et al., 2008). Functional differences in pain-modulation circuitry likely underlie differences in opioid analgesia but, it is unknown how differences in PAG-RVM manifest in terms of RVM physiology. Considering RVM is the final output of this system and RVM cell activity sets the nociceptive “tone”, it is important to determine if there are differences in RVM activity.

1.4.2 RVM cell activity in female animals

Importantly, the information presented in section 1.2, characterizing RVM physiology in baseline and during pain states, is from studies in male animals. While there has been increased interest in sexual dimorphisms in PAG projections to RVM, there has been very little work in the RVM itself. Understanding if there are differences in RVM cell activity, the physiological output of the system, is important to fully characterizing sexual dimorphisms in pain-modulation systems.

As far as I am aware, only two prior studies have performed *in vivo* single-cell recording in RVM cells in female animals. In the first study (Rojas-Piloni et al., 1998), after identifying RVM cells in female animals based on response to noxious heat, the effect of vaginal stimulation was tested. Pronociceptive ON-cell ongoing activity was decreased and antinociceptive OFF-cell ongoing activity was increased, while NEUTRAL-cells did not respond. Importantly, this study also showed that vaginal stimulation increases thermal withdrawal latency, as would be expected based on changes in ON- and OFF-cell ongoing activity. In the second study (Craft et al., 2004b), the effects of oestradiol supplementation on RVM cell response to noxious heat in ovariectomized female rats was tested. ON- and OFF-cell noxious heat-evoked changes in firing were decreased in oestradiol-supplemented females compared to ovariectomized females with no supplementation. However, there was no significant difference in ongoing firing rates. As would be expected, based on similar ongoing firing rates, there was no significant difference in tail withdrawal latency. The dampened ON-cell burst and OFF-cell pause could decrease sensitivity to subsequent stimuli but more work is needed to know the implications of this finding.

While these studies show there are ON-, OFF-, and NEUTRAL-cells in RVM of female animals, how the responses compare to RVM cells in males was not examined,

nor was the effect of injury. RVM cell activity sets the tone for nociceptive transmission and a shift in the balance between ON- and OFF-cell activity can lead to enhanced or diminished pain perception (Heinricher et al., 2009). Differences in RVM cell activity between males and females could, therefore, lead to subtle differences in sensitivity or predispose females to chronic pain conditions.

1.5 Photophobia

Many chronic pain patients report multisensory hypersensitivity including sensitivity to somatic and non-somatic stimuli (Martenson et al., 2016, Wilbarger et al., 2011, Okeson et al., 2011, Freeman et al., 2009, Gutrecht et al., 1994, Lovati et al., 2015, Zanchin et al., 2007). One manifestation of this generalized hypersensitivity is sensitivity to visual light, termed photophobia. While photophobia is sometimes referred to as “photosensitivity”, because photosensitivity is a term that is commonly used in dermatology, in this thesis, aversion to visual light will be referred to as photophobia. Originally photophobia was defined as normal levels of light causing pain in the eye (Lebensohn 1934), while dazzle was the term applied to an uncomfortable, but not painful, sense of excessive brightness. More recently these terms have been combined and photophobia is more broadly defined to include any of three primary symptoms; (1) an avoidance of light due to pain in the eye, (2) exacerbation of headache or other pain by light, and (3) a generalized sense of discomfort resulting in avoidance of light with or without overt pain (Noseda et al., 2011, Noseda et al., 2018, Digre et al., 2012). In the following sections, disorders commonly associated with photophobia are discussed, and then possible neural mechanisms are addressed.

1.5.1 Clinical presentation of photophobia

Photophobia symptoms are present in many diseases including eye disorders, neurological disorders, chronic pain conditions, and psychiatric disorders (Digre et al.,

2012, Katz et al., 2016, Main et al., 1997, Freeman et al., 2009, Martenson et al., 2016, Seidel et al., 2017, Bossini et al., 2009, Gutrecht et al., 1994, Jerath et al., 2011). Symptoms manifest differently between disorders, but even within one disorder patients have different reports of what symptoms are occurring (Wu et al., 2017), making photophobia difficult to diagnose and treat.

Eye disorders that cause dysfunction of orbital and visual pathways are divided into two classes, based on anatomy of the eye. Anterior segment diseases affect the anterior third of the eyeball and include damage or disease of structures such as the cornea or iris and dry eye. In these disorders, normal levels of light often cause eye pain, which is thought to be due to primary afferent sensitization (Digre et al., 2012). Posterior segment diseases affect the posterior third of the eye including structures that lack nociceptive sensory afferents, such as the retina. In these disorders photophobia frequently presents as either “day-blindness” or perceived flashes of light, as well as abnormal sensitivity to light (Zervas et al., 1987, Simunovic et al., 1998, Prokofyeva et al., 2011). As with the rest of the diseases presented below, since primary afferents are unaffected in posterior disorders, there must be dysfunction independent of the ocular nociceptors.

Neurological conditions including blepharospasm, chiasmal compression, trigeminal neuralgia, and primary and secondary headache disorders commonly present with photophobia (Judd et al., 2007, Nath et al., 2003, Cooper et al., 2009, Digre et al., 2012, Logan et al., 2008, Kupila et al., 2006, Gutrecht et al., 1994, Kawasaki, 2012). In blepharospasm, patients experience muscle spasms and involuntary closure of the eye, accompanied by sensitivity to light during and outside of spasm. Additionally, in some patients, light can induce spasms (Judd et al., 2007, Adams et al., 2006). Sensitivity to light also occurs outside of and during attack in chronic headache patients, where light can trigger or exacerbate headache, or is generally perceived as “too” bright (Vincent et

al., 1989, Main et al., 1997, Vanagaite et al., 1997, Drummond et al., 1993, Vingen et al., 1998, Wu et al., 2017, Digre et al., 2012). Photophobia has been documented in primary headaches such as migraine and cluster headaches, as well as secondary headaches such as in meningitis, subarachnoid hemorrhage, and traumatic brain injury (TBI).

Many migraine patients experience unilateral head pain but have sensitivity to light impinging on either eye and light can cause headache to spread (Vanagaite et al., 1997, Noseda et al., 2016). Additionally, pressure pain thresholds in patients with migraine decrease during light exposure and migraineurs experience increased tenderness in neck muscles (Kowacs et al., 2001, Noseda et al., 2016). Interestingly, in patients with TBI, photophobia remains even after initial injury has healed (Bohnen et al., 1991, Truong et al., 2014, Magone et al., 2014), indicating ongoing peripheral input is not necessary for the maintenance of photophobia. These findings indicate that photophobia may be a marker of central sensitization, or changes in pain circuits in the central nervous system that lead to hypersensitivity and widespread pain and discomfort in chronic pain disorders. Photophobia symptoms are also present in other pain conditions associated with central sensitization that may have similar underlying mechanisms to migraine. However, photophobia is a defining symptom of migraine, with 80-90% of migraineurs afflicted (Wu et al., 2017), and thus photophobia has been best studied and characterized in migraine patients and largely understudied in other conditions (Noseda et al., 2018).

Multisensory hypersensitivity is well-documented in fibromyalgia and these patients have a lower discomfort threshold to visual light than healthy controls (Wilbarger et al., 2011, Martenson et al., 2016). Additionally, light can trigger or worsen periocular pain or headache in whiplash patients (Freeman et al., 2009). There are also reports of abnormal sensitivity to light in psychiatric disorders such as panic disorder or

agoraphobia (Bossini et al., 2009, Kellner et al., 1997). Thus, trigeminal input may not be necessary for the development of photophobia, providing additional support that some forms of photophobia may reflect central sensitization. Therefore, while photophobia is best documented in disorders affecting the craniofacial tissues, and especially migraine, it is likely that there are central mechanisms beyond the trigeminal system that mediate photophobia.

1.5.2 Ascending neural mechanisms of photophobia

Historically, many physicians and scientists believed that aversion to light in the absence of an eye disorder was an inorganic, psychological symptom. It is now recognized that photophobia has a neurological basis, but the exact mechanisms are largely unknown. The neural pathways through which light produces aversion and pain likely involve plasticity in central pain-transmission circuitry (Digre et al., 2012, Noseda et al., 2018, Woodhouse et al., 1993, Lovati et al., 2013, Noseda et al., 2017). It is also likely that different presentations of photophobia are due to different neural mechanisms (Noseda et al., 2011). Current proposed mechanisms of how light accesses pain-transmission pathways to cause photophobia are outlined below. These mechanisms focus on sensitization in ascending pathways at the level of primary nociceptors, second-order trigeminal nucleus neurons, or third-order thalamic neurons. Most mechanisms are researched and framed in the context of migraine, but as mentioned above, there is likely cross over into other chronic pain conditions and neurological disorder.

Firstly, it is important to note that nociceptors do not directly respond to light energy, so light must be able to access pain-transmission pathways either by indirectly activating nociceptors or through a convergence between the visual pathways and somatosensory pathways. Early explanations of photophobia focused on the former. It was postulated

that photophobia was caused by activation and irritation of primary afferent nociceptors within the eye (Lebensohn, 1951). This theory posits that, since light causes vasodilation of orbital vessels and iris constriction, and these structures are closely associated with nociceptors, nerve endings sensitized during disease could be activated by normally innocuous reflexes to light. While this is a potential explanation for exacerbation of eye pain by light in diseases afflicting the anterior segment of the eye, primary afferent sensitization would not explain photophobia in other diseases where primary ocular nociceptors are unaffected.

In headache and other craniofacial disorders, ongoing primary afferent input from the inflamed or injured tissues could lead to sensitization of second order neurons in the trigeminal nucleus (Burstein et al., 1998). Interaction of light-input and somatosensory-input at the level of the trigeminal nucleus could amplify pain transmission. Evidence for this hypothesis is that the trigeminal nucleus contains neurons that are activated by acute exposure to bright light. Activation is dependent on orbital sensory neurons being activated by increased parasympathetic outflow and vasodilation, as well as input from accessory visual pathways mediated by the olivary pretectal nucleus (Okamoto et al., 2009, Okamoto et al., 2010). Importantly, light responsive trigeminal nucleus neurons also receive input from mechanosensitive primary afferents (Okamoto et al., 2010). Therefore, if second-order neurons in the trigeminal nucleus are sensitized by craniofacial pain and have increased excitability, then convergence of light and somatosensory information could result in discomfort or exacerbation of existing pain. Sensitization and interaction in the trigeminal nucleus could contribute to photophobia experienced during cranial inflammation but would not explain photophobia in extracranial pain disorders or in conditions where craniofacial pain has resolved.

An additional hypothesis is that light could access pain circuitry through direct input from the visual pathway to higher-order pain-transmission structures such as the thalamus. Light input most likely reaches higher structures via the non-image forming visual pathway. In this pathway, photoreception occurs via intrinsically photosensitive retinal ganglion cells (ipRGC's), a subset of retinal ganglion cells that contain melanopsin and thus are photoreceptive independently of rods and cones. ipRGCs transmit information about illuminance directly to higher brain structures, including the lateral geniculate nucleus, the suprachiasmatic nucleus, and olivary pretectal nucleus, that are important in light-dependent biological functions such as circadian rhythms and pupillary light reflex (Hattar et al., 2002, Brown et al., 2010).

One line of evidence that the non-image forming pathway is critical to photophobia is that blind migraineurs with significant deficits in image-forming perception due to rod and cone photoreceptor degradation, still experience photophobia as long as the optic nerve is intact (Noseda et al., 2010). These patients have normal sleep patterns and pupillary light reflexes, showing that illuminance detection occurs, which is likely mediated by ipRGC's. However, if the non-image forming pathway is lost, patients lose the pupillary light reflex and experience irregular sleep patterns, but also do not experience photophobia. Behavioral studies in rodents also show ipRGC's are critical to light aversion (Johnson et al., 2010, Semo et al., 2010). Although the photopigment in ipRGC's is primarily responsive to blue light, it has been found that light with wavelengths outside of the blue spectrum can also cause discomfort in patients with normal vision (Main et al., 2000, Noseda et al., 2016, Matynia et al., 2016). Thus rods and cones likely contribute to photophobia as well.

ipRGC's also project directly to the posterior thalamus, lateral thalamus, intergeniculate nucleus of the thalamus, hypothalamus, amygdala, and PAG, all of which

are areas associated with somatosensation, pain, and emotion (Noseda et al., 2010, Hattar et al., 2006, Noseda et al., 2016). These pathways may provide a means for visual light to directly engage pain networks. In regard to pain-transmission pathways, the posterior thalamus is the location where second- and third-order nociceptive neurons synapse, and thalamic neurons are sensitized by dural inflammation (Burstein et al., 2010). Using single-unit recording, it was shown that a subset of trigeminovascular neurons in the thalamus that relay nociceptive signals from the dura to higher brain structures are also responsive to light (Noseda et al., 2010). Mapping also showed that dura/light-sensitive thalamic neurons project to the primary somatosensory cortex and areas of the visual cortex (Noseda et al., 2010). Thus, convergence and amplification of photic and nociceptive information on sensitized thalamic projection neurons could cause light to be perceived as too bright through connections with the visual cortex or exacerbate existing pain through connections with the somatosensory cortex (Noseda et al., 2011).

The mechanisms outlined above are “bottom-up” explanations for photophobia, whereby light is acting to amplify pain-transmission through ascending pathways. However, there is dysfunction in pain-modulation circuitry in conditions associated with photophobia (Vincent et al., 1989, Kowacs et al., 2001), and pain-modulation systems are responsive to light (Martenson et al., 2016). Therefore, while ascending mechanisms offer explanations of how light reaches pain-transmission pathways, it is likely that plasticity in descending pathways also contributes to photophobia.

1.5.3 Engagement of descending pain-modulation circuitry by light

Similar to pain research in general, proposed mechanisms of photophobia have focused on sensitization of ascending pathways, and very little research has been performed to elucidate the contribution of descending pain-modulation systems. We

know there is plasticity in descending modulatory circuitry that has an important contribution during migraine and other chronic pain conditions associated with photophobia (Edelmayer et al., 2009, Heinricher et al., 2009). Importantly, RVM cell activity, the output of the pain-modulation system, is altered by light, providing a functional means for light to modulate pain (Martenson et al., 2016). Therefore, it is possible that increased descending facilitation or decreased descending inhibition during light exposure contributes to hypersensitivity.

In naïve, male rats, ambient visual light evokes changes in firing in a subset of RVM ON- and OFF-cells, such that ON-cell activity increases and OFF-cell activity decreases or ceases (Figure 3) (Martenson et al., 2016). Since ongoing activity of ON- and OFF-cells sets the nociceptive threshold and there is increased vigilance during OFF-cell pause and ON-cell burst (Ramirez et al., 1989, Heinricher et al., 1989), activation of pro-nociceptive ON-cells and suppression of anti-nociceptive OFF-cells by light could provide a means for ambient light to modulate pain transmission. In fact, in naïve animals, light exposure produces small but measurable thermal hyperalgesia (Martenson et al., 2016).

While Martenson et al., 2016 showed that RVM pain-modulating neurons in rats respond to light, all work was conducted in naïve males utilizing one, relatively high, light intensity. Therefore, one goal of this thesis was to establish stimulus-response curves in male and female animals and to determine if the response is altered in a state of peripheral inflammation. Testing the effects of light in male and female animals is important because clinical findings show that chronic pain disorders are more prevalent in females and differences in RVM output could predispose females to chronic pain disorders. RVM cell responses to light during injury is of interest because RVM response to somatic stimulation is enhanced during persistent peripheral pain (Cleary et al., 2013,

Carlson et al., 2007) and RVM activity is also critical to the development of cutaneous allodynia in migraine (Edelmayer et al., 2009). Thus, it is reasonable to suspect that RVM contributes to photophobia. This could be through recruitment of additional RVM cells to the photoresponsive population or by enhanced photoresponsiveness of individual RVM cells, but could have widespread effects on pain threshold.

As noted in section 1.5.2, nociceptors do not respond directly to light energy, and Martenson et al., 2016 showed that RVM response to light is independent of the trigeminal ganglion. Therefore, the effects of light on RVM cell activity may be through a "top-down" mechanism, by engagement of higher relays that ultimately affect RVM output. One possible pathway is through the olivary pretectal nucleus (OPt). RVM response to light is attenuated if OPt is blocked (Martenson et al., 2016). Further, OPt receives projections from ipRGC's (Hattar et al., 2006, Morin et al., 2003, Langel et al., 2015), thus providing a means for visual light to reach RVM.

Light-evoked changes in RVM activity could exacerbate headache due to increased facilitatory and decreased inhibitory output to the trigeminal dorsal horn. Further, during ocular disorders, similar changes in RVM activity could lower the thresholds of nociceptors innervating the orbit, resulting in ocular pain during exposure to innocuous levels of light. Additionally, because individual RVM neurons project diffusely to the trigeminal dorsal horn and multiple spinal levels, the effects of light on RVM cell activity could produce widespread changes in nociceptive sensitivity leading to generalized somatic discomfort as in fibromyalgia or whiplash injury (Huisman et al., 1981, Skagerberg et al., 1985, Heinricher et al., 1989). Thus, enhanced RVM cell responses to light during persistent pain could cause visual sensitivity to light, as well as widespread somatic hypersensitivity.

1.6 Summary and Specific Aims

Many chronic pain patients report abnormal sensitivity to multiple sensory modalities, including light. There is dysfunction in central pain circuitry in these disorders that likely leads to photophobia. It has been shown that brain regions normally implicated in nociceptive transmission and modulation respond to visual light. Specifically, a subset of neurons in RVM, the output node of the most well-known pain-modulating circuit, respond to bright, visual light in a pro-nociceptive manner in naïve, male animals. RVM cell activity can directly modulate behavioral sensitivity and individual RVM neurons project diffusely to multiple spinal levels, (Huisman et al., 1981, Skagerberg et al., 1985) which means that recruitment of these cell populations can produce widespread changes in nociceptive sensitivity. Given ON-cells exert a net pro-nociceptive effect and OFF-cells exert a net anti-nociceptive effect, activation of ON-cells and suppression of OFF-cell firing by light could provide a means for a visual stimulus to access pain-modulation pathways. Further, RVM is plastic and responses to somatic stimulation are enhanced during persistent inflammation, so plasticity in RVM could contribute to photophobia in persistent pain states. Therefore, the overall goal of this thesis was to establish stimulus-response curves for RVM cell responses to light, and to determine if response to light is altered during peripheral inflammation.

1.6.1 Aim 1: Establish stimulus-response curves for RVM cell response to visual light exposure in male and female rats

The focus of the first aim of my thesis was to characterize RVM cell activity in female animals, because a major gap in our knowledge of RVM physiology is that the activity of these pain-modulating neurons has not been well-characterized in female animals. There is a substantial but contradictory literature in humans and animals on pain sensitivity. Nevertheless, it seems that chronic pain disorders are more prevalent in females, and it has been established that there are molecular and structural sexual

dimorphisms in pain modulation-circuitry. However, physiology and function of RVM activity has yet to be compared in the sexes. In fact, only a few studies have performed *in vivo* single-cell recording in RVM in female animals, and these studies did not make direct comparisons to males. It is important to quantify RVM output, as differences in pain modulation could predispose females to develop chronic pain conditions. In aim 1, I established stimulus-response curves for RVM response to light in naïve male and female animals.

1.6.2 Aim 2: Determine if peripheral inflammation shifts the stimulus-response curves for RVM cell response to visual light in male and female rats

The focus of the second aim of my thesis is to determine if inflammation enhances photoresponsiveness of RVM cells. RVM is an important contributor to both normal and clinically significant pain. In models of hindpaw inflammation or nerve injury, ON- and OFF-cells in RVM are “sensitized,” and demonstrate lowered thresholds for responses to cutaneous stimulation. Thus it is reasonable to suspect that RVM cells may also be sensitized to light during persistent inflammation. Recruitment of additional cells to the photoresponsive population or more robust response of individual cells to light during persistent inflammation could contribute to photophobia. In aim 2, I established stimulus-response curves for RVM cell response to light in male and female animals with peripheral inflammation. My hypothesis is that RVM neurons will have an enhanced response to light but that the response will be more greatly enhanced in females than males.

1.6.3 Significance and Innovation

Sex differences in pain sensitivity have been the focus of many recent reviews but remain a debated and controversial topic. However, it seems clear that functional pain disorders are more prevalent in females, but potential, slight differences in pain

sensitivity are likely not robust enough to increase the likelihood of females developing chronic pain conditions. Moreover, studies showing no differences in nociceptive sensitivity in the sexes have found sexual dimorphisms in pain-circuitry. It is possible that under basal conditions these qualitative differences have similar behavioral endpoints but differ in response to injury. Therefore, increased knowledge of sexual dimorphisms in molecular, cellular, and circuitry aspects of pain is essential if we are to fully understand chronic pain and develop better treatments. The following studies examined RVM physiology during baseline and in persistent peripheral inflammation in males and females to understand how modulation of descending control could contribute to sex differences in pain experience and prevalence of chronic pain.

Further, dysfunction of descending pain-modulating systems is difficult to verify in individual patients, where invasive recording studies are not possible. If my findings reveal that pain-modulating neurons become sensitized to light during peripheral inflammation, photophobia in patients could be used as a clinical indicator of central sensitization.

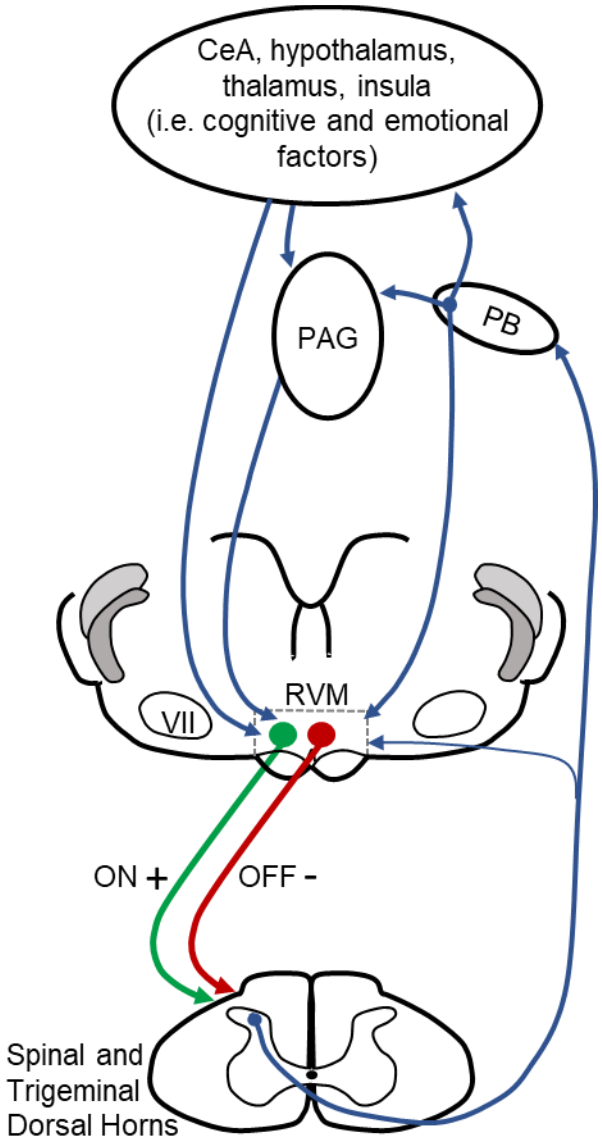

Figure 1 Nociceptive transmission is modulated by RVM

The rostral ventromedial medulla (RVM) is the final output of a pain-modulation circuit and exerts pain facilitatory or inhibitory effects via ON- and OFF-cells, respectively. This bidirectional system is also influenced by cognitive and emotional factors by “top-down” inputs from higher structures.

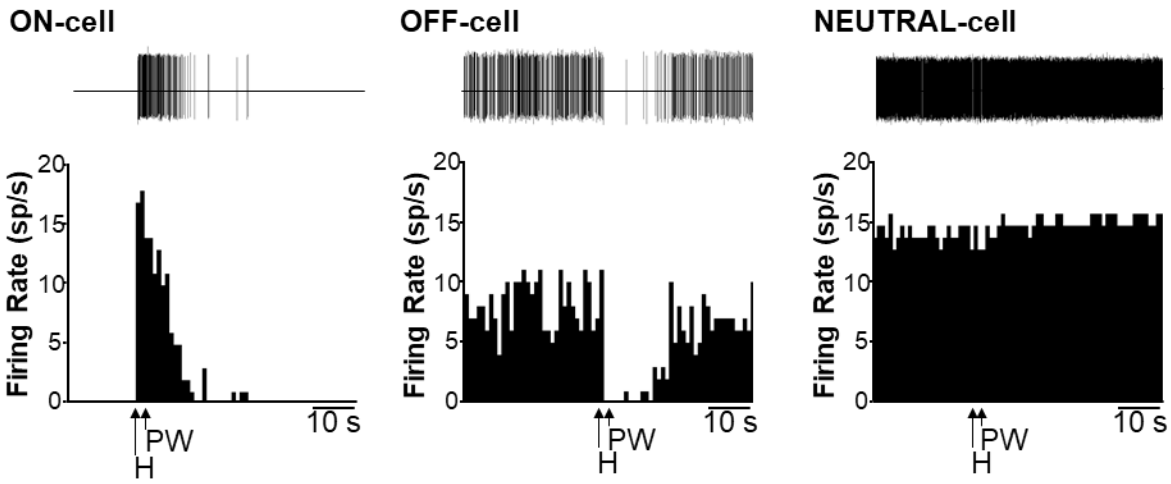

Figure 2 RVM cells are characterized by their response to noxious stimuli

Top panels show individual action potentials and bottom graphs show ratemeter records (1-sec bin) from an ON-cell (left), OFF-cell (middle), and NEUTRAL-cell (right) during noxious heat-evoked paw withdrawal. ON-cells are activated by noxious heat and have a “burst” in activity, while OFF-cells show a “pause” in any ongoing activity just prior to paw withdrawal. NEUTRAL cells do not exhibit a change in firing associated with noxious stimulus-evoked paw withdrawal.

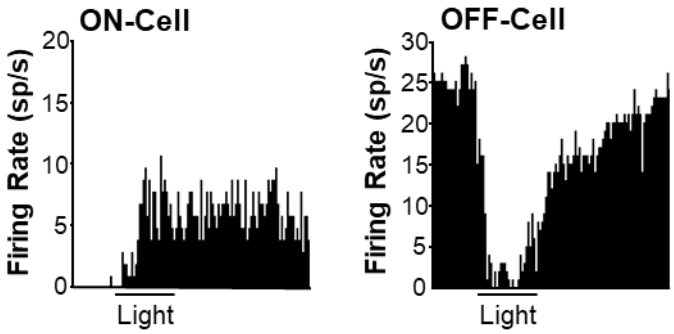

Figure 3 Light-evoked RVM cell response

Ratemeter traces show representative ON-cell (left) and OFF-cell (right) response to bright light with 30-s light stimulation noted below. A subset of RVM ON-cells (left) and OFF-cells (right) are activated and suppressed, respectively, by bright light shined in the eye.

CHAPTER 2

MANUSCRIPT #1

Characterization of RVM cell activity in female animals

Gwen M. Hryciw and Mary M. Heinricher

2.1 Abstract

Although functional pain disorders disproportionately impact females and there are sex-related differences in severity of symptoms, most pain research in animals has been conducted in males. Recent evidence shows there are molecular sexual dimorphisms in pain-modulation circuitry, but very little work has been done to characterize the physiology of the output of the system, the rostral ventromedial medulla (RVM). Sex-related differences in RVM output could predispose females to pathological pain. The goal of this study was to determine ongoing cell activity, noxious stimulus-evoked responses, and light-evoked stimulus-response curves in female compared to male rats. Light is of particular interest because, in males, a subset of RVM cells is responsive to visual light. While many chronic pain patients report abnormal sensitivity to light ("photophobia"), how light-related input engages pain processing is unknown. Convergence of light-related input with pain modulation in RVM could contribute to photophobia. We found that RVM neuronal responses to noxious heat and visual light are similar in the sexes. Additionally we found that RVM cells respond to very low levels of light, and that the responses to light are graded with stimulus intensity. These data show that, while there are potential sex differences in pain-modulation circuitry upstream of RVM, the overall tone and output of the descending pain-modulation system is comparable. Our findings also argue for differences in how light and somatic stimuli engage RVM, and suggest that light-related information represents a "top-down" input to RVM.

2.2 Significance Statement

Photophobia is a common complaint in functional pain disorders, but the underlying mechanisms are largely unknown. In this paper we further characterize a potential mechanism through which light could engage pain-modulation circuitry. Additionally, we

demonstrate that activity of identified pain-modulating neurons in RVM is similar in male and female animals.

2.3 Introduction

Chronic pain disorders disproportionately impact females, but studies in healthy humans indicate that while there are likely few sex differences in pain sensitivity, males and females may experience pain differently (Fillingim et al., 2009, Mogil et al., 2000, Racine et al., 2012a). This indicates that there are likely sex differences in pain processing which could be due to sexual dimorphisms in pain circuitry. However, most pain circuitry research in animals is conducted in males (Mogil et al., 2005), but recent evidence shows there are structural and molecular sexual dimorphisms in pain-modulation circuitry (Boyer et al., 1998, Loyd et al., 2006). The rostral ventromedial medulla (RVM) is the functional output of this pain-modulating circuit, and has been well-characterized in male animals. It is possible that there are sex differences in RVM activity, but only a few studies have performed *in vivo* single-cell recording in RVM in females (Craft et al., 2004b, Rojas-Piloni et al., 1998). Moreover, these studies did not make direct comparisons to males. If we are to fully understand pain in females, we cannot assume that the physiology of the RVM is the same in both sexes. The purpose of this study was to characterize the physiology of RVM cells in female compared to male animals.

The RVM modulates nociceptive transmission through its projections to the spinal and trigeminal dorsal horns. Two classes of RVM neurons, termed “ON-cells” and “OFF-cells”, have been identified physiologically: activity of ON-cells increases (or “bursts”), whereas activity of OFF-cells ceases (or “pauses”) prior to behavioral responses to noxious stimuli (Fields et al., 1983a). These two cell classes, respectively amplify and suppress nociceptive transmission, producing hyperalgesia or hypoalgesia (Heinricher et al., 2009, Heinricher et al., 2013). Other neurons in RVM are referred to as “NEUTRAL-

cells," and whether they have a role in pain modulation is unknown. RVM also receives information from higher structures, providing a circuit through which cognitive and emotional factors can influence pain (Heinricher et al., 2013). This pain-modulation circuitry is an important contributor to both normal and pathological pain, and while well-characterized in males, little is known about its activity in females (Heinricher et al., 2009, Heinricher et al., 2013, Porreca et al., 2001, Ren et al., 2002).

It is important to compare RVM output between the sexes because a shift in the balance between ON- and OFF-cell activity can lead to enhanced or diminished pain perception (Heinricher et al., 2009). We first compared noxious heat-evoked responses, because noxious heat is the most reliable way to classify RVM cells, and the duration of response to noxious stimuli corresponds to a time of increased sensitivity where subsequent stimuli can evoke a response at a lower threshold (Fields et al., 1983a, Ramirez et al., 1989). Next we compared RVM cell ongoing activity, because ongoing activity sets the "tone" for nociceptive transmission at stimulus onset, modulating the behavioral withdrawal threshold (Heinricher et al., 1989).

Additionally, since a subset of RVM cells in males respond to visual light (Martenson et al., 2016), we established and compared stimulus-response curves for RVM cell light-evoked activity. Many chronic pain patients, such as those with migraine or fibromyalgia, report abnormal sensitivity to light, but the neural pathways through which light evokes discomfort are unknown (Main et al., 1997, Woodhouse et al., 1993, Zanchin et al., 2007, Martenson et al., 2016, Gutrecht et al., 1994, Wilbarger et al., 2011, Katz et al., 2016). Additionally, sex-related differences in severity of symptoms such as photophobia have been reported in migraine (Buse et al., 2013, Lipton et al., 2001a, Bolay et al., 2015, Zanchin et al., 2007). RVM response to light is independent of primary sensory transmission and likely represents a "top-down" input that modulates RVM cell activity (Martenson et al., 2016). Given ON-cells exert a net pro-nociceptive effect and OFF-cells

exert a net anti-nociceptive effect, activation of ON-cells and suppression of OFF-cell firing by light could provide a means for ambient light to modulate pain transmission. Differences in RVM response to light could lead to differences in the pain experience between males and females.

In summary, the focus of this study was to characterize the physiological output of the descending pain-modulation system in females by examining RVM cell activity and responses to noxious somatic input, as well as visual light. We found the basic characteristics of RVM cell activity are similar between the sexes. We also established stimulus-response curves to light that demonstrate RVM cell response is similar between the sexes. Additionally, dim levels of light can evoke a response from some RVM cells, and response to light is graded with stimulus intensity.

2.4 Methods

All experiments followed the guidelines of the National Institutes of Health and the Committee for Research and Ethical Issues of the International Association for the Study of Pain, and were approved by the Institutional Animal Care and Use Committee at the Oregon Health & Science University.

2.4.1 Lightly Anesthetized Preparation

Age-matched male and female rats (weight, 230-370 g and 170-300 g, respectively) from Charles River were used in all experiments. Animals were housed in 12 hr dark/12 hr light cycle and all experiments were performed during the light phase. Following previously described methods (Cleary et al., 2013, Martenson et al., 2016), animals were anesthetized using 4% isoflurane and a catheter placed in the external jugular vein for subsequent infusion of methohexital. Animals were then transferred to a stereotaxic apparatus and kept deeply anesthetized while a small craniotomy posterior to the lambda suture was drilled to gain access to RVM. After surgery, anesthesia was adjusted so that the animal withdrew its hindpaw to noxious heat exposure but did not

display spontaneous movement. Animals were maintained at this stable anesthetic plane for the duration of the experiment by infusion of methohexital at a constant rate. Heart rate and body temperature were monitored, and experimental protocol was initiated once these were stable and the methohexital flow rate had not been adjusted for a minimum of 45 min. Anesthetic rate was not adjusted for the rest of the protocol. Males required a higher anesthetic rate than females ($t(100)=18.74$, $p<0.0001$, $n=102$; M: 88.77 +/- 7.85 mg/kg/hr, F: 61.09 +/- 7.07 mg/kg/hr) to achieve a similar anesthetic depth based on nociceptive withdrawal (Merkel et al., 1963). All testing was performed in low ambient light conditions (<5 lux) and the pupils were dilated to eliminate differences in amount of light reaching the retina due to pupillary light-reflexes.

2.4.2 Electrophysiological Recording

A gold- and platinum-plated stainless-steel microelectrode was placed in the RVM to record cell activity. Signals were amplified and band-pass filtered (Neurolog, Digitimer) then transmitted to a computer for real-time spike detection and monitoring using Spike2 software (Cambridge, UK). Identified neurons were classified as ON-, OFF-, or NEUTRAL-cells, as originally defined (Fields et al., 1983a, Cleary et al., 2013, Martenson et al., 2016) based on changes in firing rate associated with noxious-heat evoked withdrawal. ON-cells are defined by a burst of activity beginning just prior to withdrawal from a noxious stimulus or, if active, and increase in activity. OFF-cells stop firing just prior to withdrawal. NEUTRAL-cell firing does not change in response to noxious stimuli. After isolating and identifying a cell, two heat trials were performed at 2.5-min intervals to determine magnitude of response to noxious heat. Next, cell response to light was tested by placing a fiber-optic light source (Dolan-Jenner Fiber-Lite; Dolan-Jenner Industries, Buxborough, MA) 5 cm from the left eye to deliver diffused light at a range of intensities in ascending order (330, 575, 900, 6,000, 10,500, and 16,000 lux). Each intensity was applied for 30-sec at 2.5-min intervals, with each

intensity repeated two times. A final heat trial was then performed to confirm anesthetic stability.

2.4.3 Histology

At the end of each experiment, the recording site was marked with an electrolytic lesion. Animals were euthanized by methohexital overdose and perfused transcardially with saline and 10% formalin. Brains were removed, and brainstems were sectioned on a Leica CM3050 S cryostat (60 µm sections). RVM lesion was photographed with an Optronics Microfire camera attached to an Olympus BX51 microscope. RVM was defined as the nucleus raphe magnus and adjacent reticular formation medial to the lateral boundary of the pyramids at the level of the facial nucleus (Paxinos et al., 2009).

2.4.4 Data Processing and Analysis

At the conclusion of each experiment, action potential waveforms were individually examined to verify correct cell identity.

Ongoing activity was defined as the average firing rate over the 30-sec periods prior to initiation of the three heat trials. Heat-evoked reflex-related neuronal activity for ON-cells was defined as the total number of spikes in the burst and the duration of the burst, where a “burst” is defined as the first action potential after heat onset until the last action potential that precedes a 2-s quiet period. If a cell was active prior to heat onset, then the number of action potentials in the 3-s period around the paw withdrawal was used to define the number of spikes in the burst and burst duration was not defined for these cells. Heat-evoked reflex-related neuronal activity for OFF-cells was defined as the percent suppression quantified by the firing rate in the 3-s period around the paw withdrawal relative to the firing rate 30 s prior to heat onset and as the duration of the pause. A “pause” was considered to begin at the last spike after heat onset that was not followed within 2 s by another action potential and lasted until there were two action potentials within 2 s of each other. Pause duration was not defined for trials were the cell

was quiet preceding heat onset. Percent pre-stimulus firing was also determined for NEUTRAL-cells based on the firing rate in the 3-s period around the paw withdrawal compared to the 30-s period preceding heat onset.

Overall a cell was considered to be “light-responsive” if it had a positive response at 16,000 lux. A positive response was one in which cell activity changed by at least 50% during light exposure relative to the 30-s period preceding light onset. In addition, for a response to be consider positive, a minimum of 10 action potentials during the stimulus was required of ON-cells, and a minimum of 10 action potentials prior to light simulation was required of OFF-cells. The percentage of cells that responded within each light intensity was also determined following the same criteria to define a response as positive. For light-responsive cells, stimulus-response curves for light-related firing activity were generated based on the average of the two trials. For ON-cells, the total number of action potentials in the burst and the duration of the burst were defined following similar criteria to define a “burst” as in heat trials. If an ON-cell was active prior to a light trial, then the total number of spikes during the light trial was used to define the number of spikes in the burst and burst duration was not quantified for the trial. For OFF-cells, the percent suppression was defined as the firing rate during light exposure relative to the firing rate in the 30-s period preceding the light trial. The longest OFF-cell pause initiated during light exposure was also defined following similar criteria to define a “pause” as in heat trials. Latency to response was also determined as defined by the time from initiation of the trial to the start of an ON-cell “burst” or OFF-cell “pause”. Latency was not determined for trials were an OFF-cell was inactive or ON-cell active prior to light. For ON-cells, the average firing rate and peak firing rate during light exposure were also determined.

Some cell parameters had a highly skewed distribution, so these parameters were either log transformed to normalize data or nonparametric tests were applied.

Comparisons between the sexes of ongoing activity and magnitude of heat response were made with Mann-Whitney test. Proportion of light responsive cells was compared between the sexes with Fischer's exact test and stimulus-response effect for proportion of light-responsive cells was determined using Chi-squared test for trend. Light-evoked response threshold was determined as the average lowest intensity required to evoke a response from light responsive cells and compared between the sexes with Mann-Whitney test. Area under the curve was determined for the magnitude of light-related cell activity responses and compared between the sexes with *t*-test. If no difference was found between the groups, groups were collapsed to determine stimulus-response relationships. A one sample *t*-test was used to determine if the average slopes of the stimulus-response curves were significantly different from zero. For all tests, $p<0.05$ was considered significant.

2.5 Results

Neurons were recorded in the RVM of lightly anesthetized male and female rats using extracellular single-cell recording methods. ON-, OFF-, and NEUTRAL-cells were sampled at random and defined based on responses to noxious heat, and then tested for responses to a range of light intensities. A total of 64 cells were recorded from 49 males, and 64 cells from 53 females (1-3 cells per animal). Cells were distributed throughout RVM in both males and females (Fig 4).

2.5.1 No differences in RVM cell ongoing firing and noxious stimulus-related responses in males and females

The first goal of this project was to validate the basic firing characteristics of RVM cells in female animals by drawing comparison to RVM cell activity in male animals. RVM ON- and OFF-cells are characterized by noxious stimulus-evoked activation and inhibition, respectively, while NEUTRAL-cells do not have a change in activity. Examples of ON-, OFF-, and NEUTRAL-cell responses to noxious heat in males and females are

shown in figure 5. Heat-evoked ON-cell burst, as defined by the total number of action potential (Fig 6a), and OFF-cell suppression, as defined by the percent firing during paw withdrawal relative to the firing rate prior to heat (Fig 6b), were not significantly different between the sexes. The duration of ON-cell burst and OFF-cell pause were also not significantly different between the sexes (ON-cell: $t(46)=1.82$, $p=0.075$; OFF-cells: $t(49)=0.77$, $p=0.45$). Additionally, comparisons of firing rates during unstimulated periods revealed no significant differences in the ongoing activity of ON-cells (Fig 6c) or OFF-cells (Fig 6d) in males and females. These findings suggest there is similar "tone" in pain-modulation systems in males and females. NEUTRAL-cells, which do not have a change in firing rate during noxious stimulus withdrawal, were observed in both males and females, and there was no significant difference in NEUTRAL-cell activity between the sexes (Fig 7).

2.5.2 No differences in proportion of cells responsive to light or light-evoked response threshold in males and females

The second goal of this project was to establish light-evoked stimulus-response curves for RVM cells in males and females. Examples of ON- and OFF-cell responses to low (330 lux) and high (16,000 lux) light intensities are shown in figure 8. Light-responsive ON-cells have a burst of activity, while light-responsive OFF-cells have suppressed ongoing activity during exposure to visual light. A change in activity of at least 50% during light exposure compared to the 30-s period prior to light onset was used to consider a cell to have a positive response during a light trial. The percentages of cells responsive at each intensity were determined. Stimulus-response curves showed that the percent of ON-cells (Fig 9a) and OFF-cells (Fig 9b) that responded to light were not significantly different between the sexes and that the proportion of cells responding at each intensity was stimulus-dependent. The light-evoked response threshold was also not significantly different between the sexes (Fig 9c, d). There were no NEUTRAL-cells

in either sex that responded to light, as with prior work in male animals (Martenson et al., 2016).

2.5.3 RVM ON- and OFF-cell responses to light are graded with stimulus-intensity in both males and females

The magnitude of light-evoked changes in cell firing were determined and compared in males and females among the light-responsive subset of cells. For this analysis, a given cell was defined as being photoresponsive if it had a positive response to 16,000 lux, the highest light intensity tested. There was a significant stimulus-response relationship for ON-cell burst, as defined by the total number of spikes evoked by light (Fig 10a), and for OFF-cell suppression, as defined by the percent firing during light exposure relative to the 30-s prior to light (Fig 10b). There was also no significant difference in ON-cell burst or OFF-cell pause between males and females (Fig 10).

We compared additional light-evoked response parameters including the ON-cell burst duration, peak firing rate, and latency to a response, and OFF-cell pause duration and latency to pause. There was a significant stimulus-response relationship between all parameters and light intensity, except for latency to response, further supporting that light-evoked responses are graded. There were no significant differences between males and females in any of these parameters (Table 1).

In sum, these results show RVM cells in males and females respond similarly to noxious heat stimulation and during exposure to visual light. RVM cell response to light was also found to be stimulus-dependent.

2.6 Discussion

The major goal of this study was to directly compare RVM cell activity, the physiological output of a major pain-modulation system, in males and females. We first considered ongoing activity and noxious heat-related responses of RVM cells, and found that RVM cells in males and females have similar firing properties. Since RVM is the

output of a major pain-modulation circuit and can amplify or suppress pain-transmission (Heinricher et al., 2009, Heinricher et al., 2013), sex-related differences in the tone and activity of this system could conceivably predispose females to develop pain conditions. However, our data show that despite structural and molecular differences in pain-modulation circuitry upstream of RVM (Boyer et al., 1998, Loyd et al., 2006, Loyd et al., 2007, Loyd et al., 2008), overall descending output from the pain-modulation system is comparable in males and females under basal conditions.

Despite sex differences in the prevalence of chronic pain disorders and potential sex differences in pain experience in humans, studies on sex differences in nociceptive sensitivity in rodents have conflicting results (Mogil et al., 2000). Sex differences are found in either direction, or not at all, and results are highly variable both between different nociceptive assays, and within a given nociceptive assay (Loyd et al., 2008, Wang et al., 2006, Boyer et al., 1998, Bradshaw et al., 2000, Terner et al., 2005, Bobeck et al., 2009, Tershner et al., 2000, Doyle et al., 2018, Doyle et al., 2018, Cook et al., 2006, Mogil et al., 2000, Greenspan et al., 2013). Results are also dependent on other methodological details such as animal strain, laboratory environment, and experimenter (Mogil, 2017, Chesler et al., 2002, Kest et al., 1999, Mogil et al., 2010, Wilder et al., 1982, Lacroix-Fralish et al., 2006). On the whole, it is unlikely that there are sex differences in nociceptive sensitivity, but rather other more important factors at play. Our data provide physiological evidence to support this assertion by demonstrating that the nociceptive tone of the pain-modulation system is not different between males and females.

We also sought to characterize RVM cell response to a non-noxious stimulus, visual light. Light is of interest because it has been shown that ambient visual light evokes a response in a subset of RVM cells in male animals (Martenson et al., 2016). However, RVM cells in female animals were not previously examined. Modulation of RVM cell

activity by light could provide a means for light to engage pain-processing systems. Consequently, basal differences in light response could lead to females being more susceptible to photophobia in chronic pain conditions (Buse et al., 2013). Additionally, because light can lower the threshold to heat-evoked withdrawal (Martenson et al., 2016), sex differences in RVM response to light could have behavioral implications. Thus, stimulus-response curves for RVM cell response to visual light were compared in males and females. We found responses between the sexes were comparable in terms of proportion of cells responding to light, light-evoked response threshold, and magnitude of response to light. Therefore, as with thermal stimulus, RVM photoresponsiveness supports that descending pain-modulation output is similar in the two sexes. This suggests that baseline differences in pain-modulation circuitry likely do not predispose females to photophobia.

RVM cells responded even to dim levels of light as reflected by at least 50% of cells responding to 330 lux, the lowest light intensity tested. These low intensities are well below the threshold shown to activate a nociceptive pathway in rats (5,000 lux) (Okamoto et al., 2010) and much lower than intensities tolerated by human subjects (Kowacs et al., 2001, Woodhouse et al., 1993). Moreover, previous findings show that RVM cell response to light is independent of the trigeminal ganglion (Martenson et al., 2016). Although the simplest mechanism to explain the RVM cell response to light would be that light is acting as a noxious stimulus and evoking a response in RVM cells via the trigeminal sensory system, this seems unlikely. Rather, these findings reflect that light and somatic stimulation likely access RVM via different pathways.

Additional support for the hypothesis that somatic stimuli and light engage RVM through different pathways is that the responses are qualitatively different. In both sexes, we found that RVM ON- and OFF-cell response to light is graded with stimulus intensity. The graded response to light and response to dim levels of light is in contrast to the

effects of somatic stimulation, in which RVM cells respond in an all-or-nothing manner and only respond to suprathreshold stimuli (Cleary et al., 2013). Nociceptive input forms a bi-directional feedback loop with RVM where somatic stimuli act as a bottom-up input to RVM, causing a change in RVM cell activity. Meanwhile RVM has descending projections that modulate sensory transmission neurons, ultimately having an effect on the behavioral response threshold (Fields et al., 1985). This positive feedback loop means that the magnitude of RVM cell response to somatic stimuli is likely to be restricted to a given quantity of action potentials. This is because cell response to noxious stimuli is bound on the lower end since only suprathreshold stimuli evoke a response, and also bound on the upper end since the animal withdraws from the stimulus. Light, on the other hand, may act as a top-down input that can influence the excitability of the pain-modulation system through graded changes in RVM cell activity.

One implication of the characteristics of the RVM response to light is that even dim light could have behavioral effects. Prior work showed that exposure to 18,000 lux, a level representative of full daylight, lowered the thermal withdrawal threshold in male rodents (Martenson et al., 2016). Other light intensities were not tested but since much lower intensities also evoke a pronociceptive response in RVM, dim light or light that is not perceived to be uncomfortable may also increase sensitivity to noxious stimuli. Furthermore, since RVM response to somatic stimuli is enhanced in persistent pain states, interaction of light-related inputs with the somatosensory system could cause allodynia and widespread discomfort. This might be the case in some chronic pain disorders where pain pressure thresholds are lowered during exposure to light (Kowacs et al., 2001).

As with prior work, we saw no effect of noxious heat or visual light on activity of NEUTRAL-cells in either sex, further supporting that NEUTRAL-cells do not have an apparent role in pain-processing (Fields et al., 1985).

In summary, we demonstrated that RVM responses to noxious heat and visual light are similar between the sexes. Additionally we showed that RVM response to light is graded with stimulus intensity. The overall tone and output of the descending pain-modulation system is thus comparable in males and females under basal conditions. RVM neurons in males develop enhanced responses to somatic stimuli in models of persistent pain (Carlson et al., 2007, Cleary et al., 2013), whether this is also seen in females has yet to be explored. However, there are sex-related differences in effects of inflammation on other parts of the pain-modulation system (Loyd et al., 2008, Loyd et al., 2006), so RVM response during injury may also differ. Overall, this work provides a foundation for the use of female animals in RVM recording work, but future work will be needed to determine if RVM cells response in females is altered during inflammation and in other pain models. Additional work will also be needed in both sexes to determine if, similar to response to somatic stimuli, RVM cell response to light is enhanced during persistent inflammation.

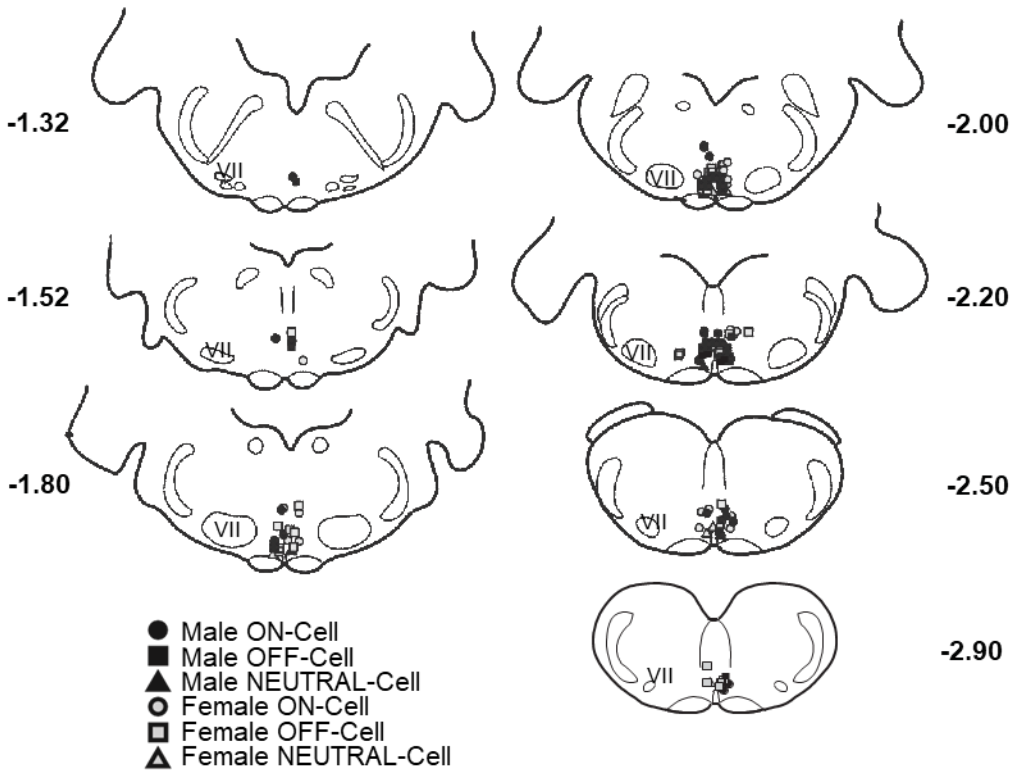

Figure 4 Recording locations within the RVM

ON-, OFF-, and NEUTRAL-cells were distributed between -1.32 mm and -2.90 mm relative to the interaural line, with the majority of the cells recorded between -1.80 mm and -2.5 mm. Cells in males and females were evenly distributed across sections. There were 29 ON-, 25 OFF-, and 10 NEUTRAL- cells recorded from 49 male animals and 28 ON-, 26 OFF-, and 10 NEUTRAL-cells recorded from 53 female animals.

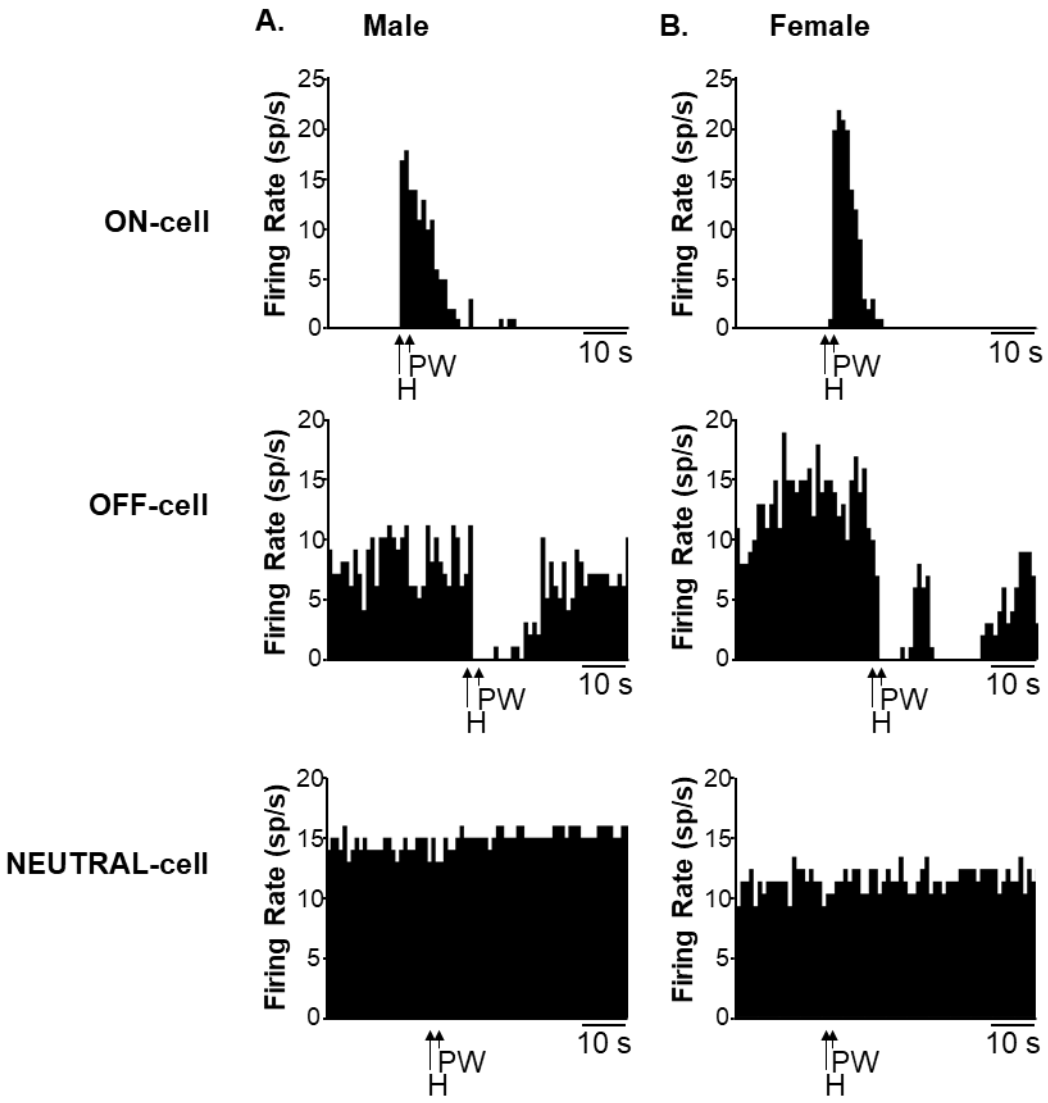

Figure 5 Representative heat-evoked responses of ON-cell (top), OFF-cell (middle), and NEUTRAL-cell (bottom)

Ratemeter records (1-s bins) show heat-evoked cell responses recorded from RVM in male (left) and female (right) rats with heat onset (H) and paw withdrawal (PW) noted below. ON-cells have a burst of activity while OFF-cells have a pause or suppression of ongoing activity just after heat onset and prior to paw withdrawal. NEUTRAL-cell firing rate does not change in response to noxious stimulation.

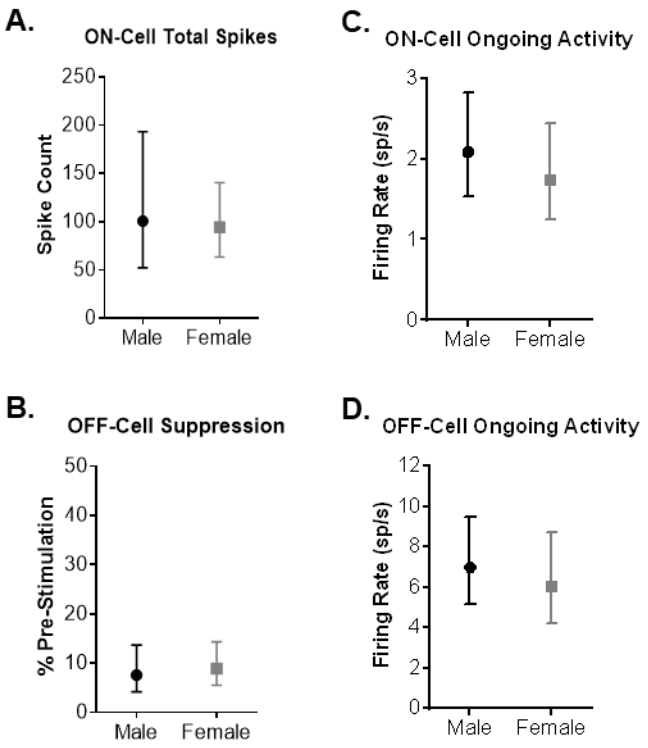

Figure 6 Noxious stimulus-related responses and ongoing activity of ON- and OFF-cells in male and female animals

Heat-evoked ON-cell burst (A), as defined by the total number of spikes in the burst, and OFF-cell suppression (B), as defined by the percent firing during paw withdrawal relative to firing prior to heat onset, was not significantly different between males and females. Average ongoing firing rates of ON-cells (C) and OFF-cells (D) during unstimulated periods were also not significantly different between males and females.

Statistical analysis: A. Mann-Whitney test, U=395, p=0.86. B. Mann-Whitney test, U=296, p=0.59. C. Mann-Whitney test, U=324.5, p=0.19. D. Mann-Whitney test, U=311, p=0.80. Data are displayed as geometric mean +/- 95% CI, n=25-29 cells/group.

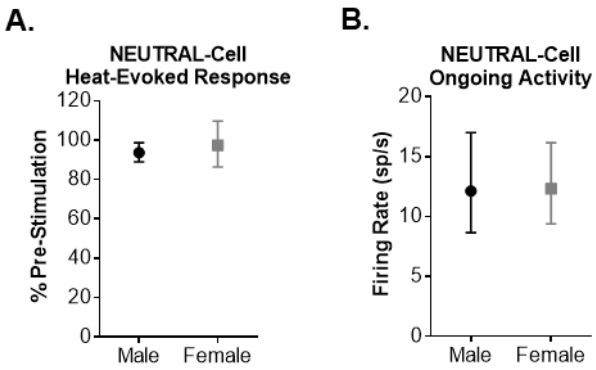

Figure 7 Noxious stimulus-related responses and ongoing activity of NEUTRAL-cells in male and female animals.

A. NEUTRAL-cell activity does not change significantly in response to noxious stimulus, as determined by the percent firing during paw withdrawal relative to firing in the 10-s period prior to heat onset. The percent pre-stimulus firing was not significantly different between males and females (Mann-Whitney test, U=45, p=0.74). B. Average ongoing firing rate of NEUTRAL-cells was not significantly different between males and females (Mann-Whitney test, U=32.5, p=0.20). Data are displayed as geometric mean +/- 95% CI, n=10 cells/sex.

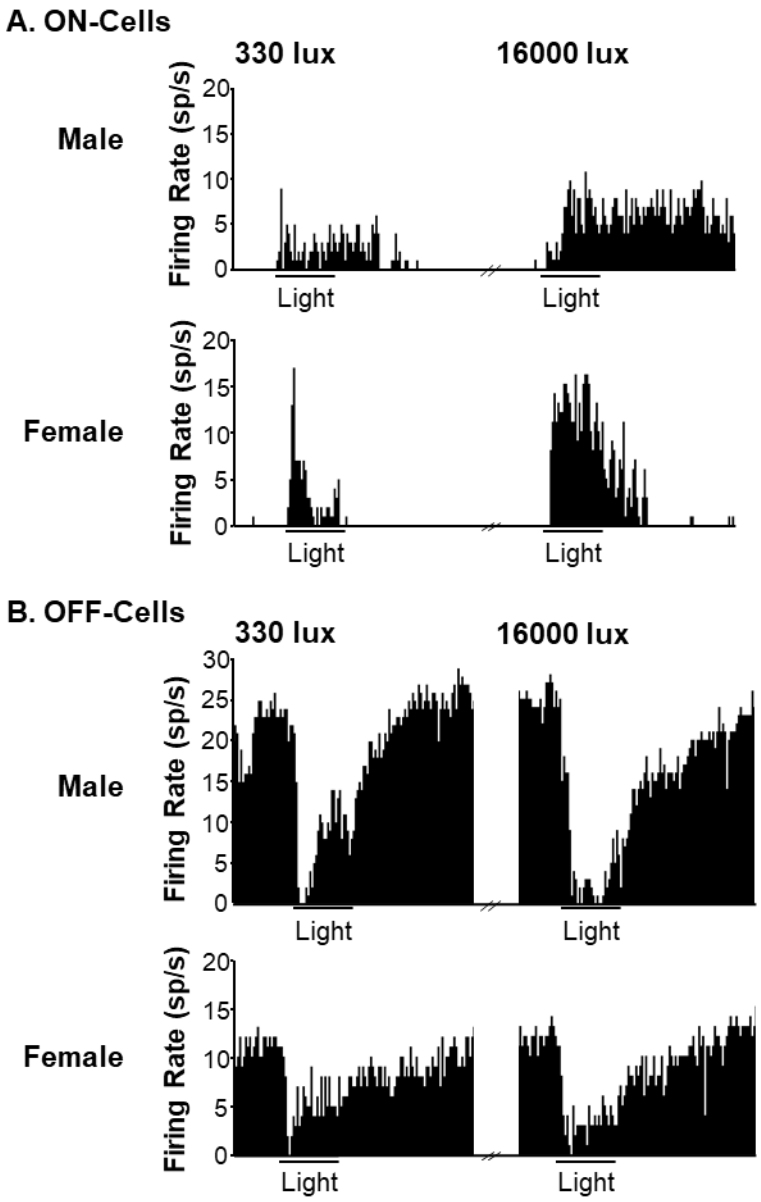

Figure 8 Representative ON-cell (A) and OFF-cell (B) responses to 330 (left) and 16,000 (right) lux

Ratemeter records (1-s bins), with the duration of light stimulus (30 s) below each trace, show examples of light-evoked responses recorded from RVM in male and in female animals during exposure to 330 and 16,000 lux. There is a clear burst in ON-cell activity and suppression of OFF-cell activity during light exposure.

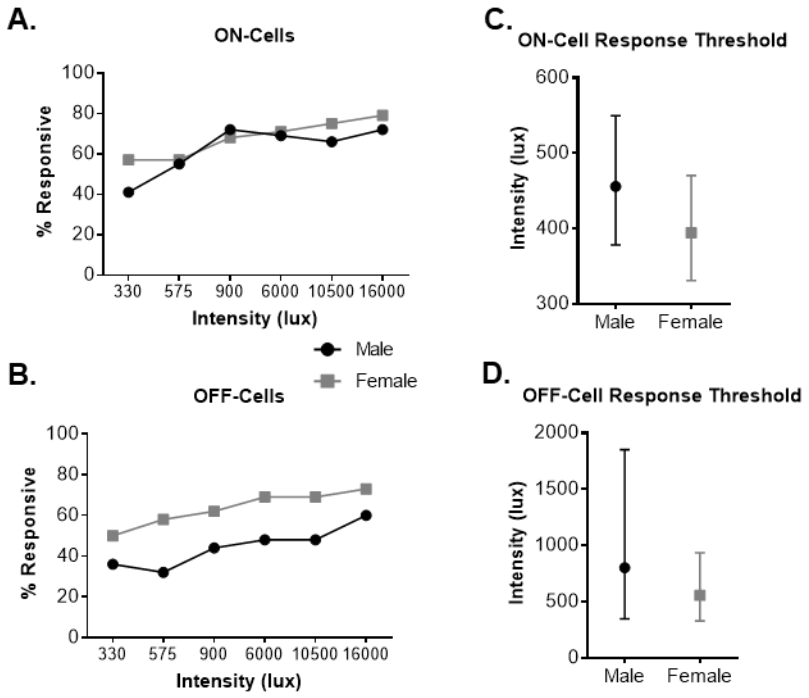

Figure 9 Proportions of RVM ON- and OFF-cells responsive to light and average response thresholds.

There is no significant difference between males and females in the overall proportion of light responsive ON-cells (A) or OFF-cells (B), as defined by the percent of cells that had a positive response at 16,000 lux. There is a significant stimulus-response effect within both cell classes. There is also no significant difference between males and females in the ON-cell (C) or OFF-cell (D) response threshold, based on the average of the lowest intensity required to evoke a positive response from the light-responsive subset of cells.

Statistical analysis proportion responsive: A. ON-cells: proportion: Fisher's exact, p=0.76, n= 29M, 28F; stimulus-response effect: Chi-squared test for trend, $X^2(1)=10.83$, p=0.0010, n= 57. B. OFF-cells: proportion: Fisher's exact, p=0.38, n= 25 M, 26 F; stimulus-response effect: chi-square test for trend, $X^2(1) = 8.76$, p=0.0031, n=51. Proportion data is displayed as the percent cells with a "positive response" at each

intensity, as defined by a change in at least 50% firing during light exposure relative to the 30-s period prior to light, n=25-29 cells/group.

Statistical analysis thresholds: C. ON-cells: Mann-Whitney test, U=167.5, p=0.19, *n= 21 M, 20 F*. D. OFF-cells: Mann-Whitney test, U=127.5, p=0.56, *n= 15M, 19F*. Thresholds are displayed as geometric mean +/- 95% CI, n=15-21 cells/group.

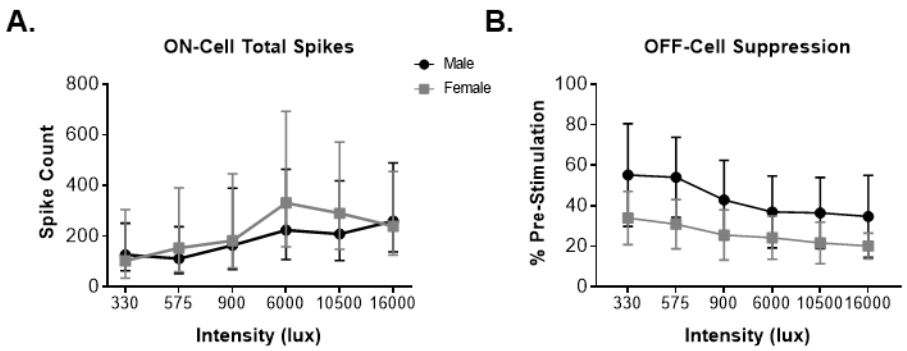

Figure 10 Light-evoked RVM ON- and OFF-cell response

There is no significant difference in ON-cell burst (A), as defined by total number of spikes, or OFF-cell suppression (B), as defined as percent pre-stimulus firing during light, between male and female animals. There was a significant effect of light intensity on cell response based on the average slope of the stimulus-response curves being significantly different from 0.

Statistical analysis: A. ON-cell burst (sex: $t(41)=0.2902$, $p=0.77$; stimulus response: $t(42)=4.40$, $p<0.0001$), data are displayed as geometric mean +/- 95% CI, n=20-21 cells/sex. B. OFF-cell suppression (sex: $t(32)=1.992$, $p=0.055$; stimulus-response: $t(33)=3.40$, $p=0.0018$), data are displayed as mean +/- 95% CI, n=15-19 cells/sex.

Cell Type	Parameter	Sex Difference?	Stimulus Response?
ON	Firing rate during light	No ($t(41)=1.25$, $p=0.22$)	Yes ($t(42)=0.467$, $p<0.0001$)
ON	Peak firing rate during light	No ($t(41)=1.33$, $p=0.19$)	Yes ($t(42)=3.78$, $p=0.0005$)
ON	Light evoked burst duration	No ($t(36)=1.42$, $p=0.16$)	Yes ($t(37)=2.69$, $p=0.011$)
ON	Latency to burst	No ($t(35)=0.68$, $p=0.50$)	No ($t(36)=0.77$, $p=0.45$)
OFF	Longest pause duration	No ($t(32)=1.10$, $p=0.28$)	Yes ($t(33)=2.06$, $p=0.047$)
OFF	Latency to pause	No ($t(32)=1.60$, $p=0.12$)	No ($t(33)=0.72$, $p=0.48$)

Table 1 Additional ON- and OFF-cell light-evoked response parameters

Other parameters that can be used to quantify ON-cell response to light include the firing rate during light exposure, peak firing rate during light, duration of burst (from time of first light-evoked spike to the first 2-s quiet period), and latency to burst (time from light onset to initiation of the burst). Other parameters that can be used to quantify OFF-cell response to light include the longest pause duration and latency to pause (time from light onset to longest pause). These parameters demonstrate that there are no significant differences between the sexes. They also further support that there is a stimulus-dependent effect of light intensity on changes in cell firing.

Note: “Light evoked burst duration” and “Latency to burst” were not calculated for cells that were active at the initiation of a light trial, so some values are missing for these parameters.

CHAPTER 3

MANUSCRIPT #2

Changes in visual light-evoked responses of brainstem pain-inhibiting neurons in persistent inflammation

Gwen M. Hryciw, Jennifer Wong, and Mary M. Heinricher

3.1 Abstract

Photophobia, a symptom common to multiple chronic pain disorders, may reflect dysfunction in pain-processing circuitry. One mechanism through which light could influence pain is through the rostral ventromedial medulla (RVM), the output node of a major pain modulation circuit. RVM is important to both normal and clinical pain, and RVM is plastic, developing enhanced responses to somatic stimuli during injury. In naïve rats, a subset of RVM cells respond to visual light. RVM cell responses to light could be altered in persistent pain and thus contribute to photophobia. Additionally, while it has been shown that there are molecular differences in pain-modulation circuitry between the sexes, few studies have compared the physiological and functional output, i.e. RVM cell activity. In this project, we compared the effects of persistent inflammation on RVM cell light-evoked stimulus-response curves in male and female animals. We found that inflammation shifted the stimulus-response curve of pain-inhibiting OFF-cells such that they are inactivated at a lower threshold and to a greater degree by dim levels of light. There was no effect on pain-facilitating ON-cells. Therefore, in pain states, decreased descending inhibition during light exposure could contribute to photophobia. Additionally, the effects of inflammation are no different in the two sexes, reflecting that despite molecular differences, there are similar endpoints in the pain-modulation system.

3.2 Perspective

Light-induced discomfort is a common complaint among patients with functional pain disorders, but the underlying mechanisms are largely unknown. In this paper we determined that persistent inflammation decreases the threshold at which pain-inhibiting output neurons in RVM are inactivated by light. This could contribute to photophobia in chronic conditions.

3.3 Introduction

Many chronic pain patients report abnormal sensitivity to multiple sensory modalities, including light. This multisensory hypersensitivity is best documented in migraine, but has also been quantified in patients with fibromyalgia (Main et al., 1997, Woodhouse et al., 1993, Zanchin et al., 2007, Martenson et al., 2016, Gutrecht et al., 1994, Wilbarger et al., 2011, Katz et al., 2016). Dysfunction of central pain-transmission and -modulation circuitry likely accounts for some of the observed photophobia (Digre et al., 2012, Woodhouse et al., 1993, Lovati et al., 2013, Noseda et al., 2017, Martenson et al., 2016). The output node of an important pain-modulating circuit is the rostral ventromedial medulla (RVM) (Heinricher et al., 2013), which contains a subset of neurons that, in naïve animals, respond to both noxious stimuli and visual light (Martenson et al., 2016, chapter 2). Thus, light could engage pain-processing systems via RVM, and plasticity in RVM could contribute to photophobia in pain states. The purpose of this study was to determine if RVM response to light is enhanced in persistent inflammation.

RVM amplifies or suppresses nociceptive transmission through two classes of neurons, termed “ON-cells” and “OFF-cells”, respectively. Activation of pain-facilitating ON-cells and suppression of pain-inhibiting OFF-cells acts as a positive feedback loop (Hernandez et al., 2001, Jinks et al., 2007), facilitating responses to subsequent inputs, and contributing to pathological pain (Heinricher et al., 2009,Ramirez et al., 1989, Heinricher et al., 2013). In models of hindpaw inflammation or nerve injury, ON- and OFF-cells are “sensitized,” and demonstrate lowered thresholds for responses to cutaneous stimulation (Cleary et al., 2013, Carlson et al., 2007). Thus it is reasonable to suspect that RVM cell responses may also be sensitized to light during persistent inflammation.

Photophobia is a broadly defined term, but can manifest as discomfort due to light being perceived as excessively bright, as a more generalized sense of discomfort, or exacerbation of existing pain (Digre et al., 2012). Engagement of RVM pain-modulating cells by light could produce widespread changes in nociceptive sensitivity, because individual RVM neurons project diffusely to multiple spinal levels to modulate nociceptive transmission and behavioral sensitivity (Huisman et al., 1981, Skagerberg et al., 1985). In naïve animals, light exposure produces small but measurable thermal hyperalgesia (Martenson et al., 2016). If additional cells are recruited to the photoresponsive population or photoresponsiveness of individual cells is enhanced during persistent inflammation, light could further lower nociceptive thresholds, contributing to widespread hypersensitivity observed in chronic pain conditions.

Despite sex differences in the prevalence and presentation of many functional pain disorders (Fillingim et al., 2009, Buse et al., 2013, Lipton et al., 2001a, Bolay et al., 2015), most pain research in animals has been conducted in males (Mogil et al., 2005). Although we have shown previously (chapter 2) that RVM cell responses to light are similar in males and females, very few *in vivo* recording studies have been performed in RVM of female animals (Craft et al., 2004b, Rojas-Piloni et al., 1998), and the effects of somatic inflammation on activity of RVM cells in females is unknown. However, we know RVM is plastic in males, and that there are molecular and structural differences in pain-modulation circuitry between the sexes (Boyer et al., 1998, Loyd et al., 2006). Thus there could also be sexual dimorphisms in RVM plasticity that could predispose females to chronic pain conditions. Therefore, in addition to determining the effects of persistent inflammation on RVM cell responses to light in both sexes, the effects of inflammation on basic firing properties of RVM cells in females were also investigated in order to compare with known effects in males (Cleary et al., 2013).

3.4 Methods

All experiments followed the guidelines of the National Institutes of Health and the Committee for Research and Ethical Issues of the International Association for the Study of Pain, and were approved by the Institutional Animal Care and Use Committee at the Oregon Health & Science University. Male and female rats from Charles River were used in all experiments.

3.4.1 Inflammation

Age-matched male and female rats weighing less than 315 and 200 g, respectively, were briefly anesthetized with Isoflurane (4%, 4-5 min) and saline (0.1 ml) or CFA (0.1 ml) was injected subcutaneously into the plantar surface of the left hindpaw. Rats were returned to their home cage for 5 to 6 days to model persistent inflammation, since inflammation peaks at this time (Ren, 1999, Ren et al., 1999). Rats were housed in 12 hr light/12 hr dark cycles, and experiments were performed during the light phase. A total of 147 ON- and OFF-cells were recorded from 115 animals (1-3 cells per animal). Twenty-three, 17, 20, and 18 ON-cells were recorded from CFA-treated males, saline-treated males, CFA-treated females, and saline-treated females, respectively. Seventeen, 19, 14, and 20 OFF-cells were recorded from CFA-treated males, saline-treated males, CFA-treated females, and saline-treated females, respectively.

3.4.2 Lightly Anesthetized Preparation

CFA injection did not have a significant effect on weight gain in males or females (Holm-Sidak's multiple comparisons, M: $t(50)=2.02$, $p=0.095$, $n=52$; F: $t(48)=0.24$, $p=0.81$, $n=50$, pre-CFA weight not obtained for some animals). Following previously described methods (Martenson et al., 2016, Cleary et al., 2013) animals were anesthetized (4% isoflurane) and a catheter placed in the external jugular vein for

subsequent infusion of methohexital. Animals were then transferred to a stereotaxic apparatus and kept deeply anesthetized while a small craniotomy posterior to the lambda suture was drilled to gain access to RVM. After surgery, anesthesia was adjusted so that the animal withdrew its hindpaw to noxious heat exposure but did not display spontaneous movement. Animals were maintained at this stable anesthetic plane for the duration of the experiment by infusion of methohexital at a constant rate. Heart rate and body temperature were also monitored. Experimental protocol was initiated once the methohexital flow rate was not adjusted for a minimum of 45 min. CFA injection did not have an effect on anesthetic requirement compared to saline injection ($F(1, 111)=0.21$, $p=0.65$, $n=115$). However, as in chapter 2, males required a higher anesthetic rate ($F(1,111)=217.6$, $p<0.0001$, $n=115$, males: 76.91 +/- 8.04 mg/kg/hr, females: 58.51 +/- 4.93 mg/kg/hr) to achieve a similar anesthetic depth (Merkel et al., 1963).

3.4.3 Electrophysiological Recording

All testing was performed in low ambient light conditions (<5 lux) and the pupils were dilated to eliminate differences in amount of light reaching the retina due to pupillary light reflexes. A gold- and platinum-plated stainless-steel microelectrode was placed in the RVM to record cell activity. Signals were amplified and band-pass filtered (Neurolog, Digitimer) then transmitted to a computer for real-time spike detection and monitoring using Spike2 software (Cambridge, UK). EMG activity, heart rate, and paw heat-stimulus temperature were also recorded using Spike2. Identified neurons were classified as ON-, OFF-, or NEUTRAL-cells, as originally defined (Fields et al., 1983a, Martenson et al., 2016, Cleary et al., 2013) based on changes in firing rate in response to noxious heat stimulation. ON-cells are defined by a burst in activity beginning just prior to withdrawal from a noxious stimulus. OFF-cells stop firing just prior to withdrawal.

After isolating and identifying a cell, ongoing activity was recorded for 10 min during which time animals were not stimulated. Stimuli were delivered at approximately 2.5-min intervals, but some trials were delayed in order to capture an ON-cell in a quiet state or an OFF-cell in an active state. Two thermal trials were performed to determine magnitude of cell response to noxious heat and latency to paw withdrawal. Noxious heat was applied by lightly resting a Peltier device (Yale Instruments, New Haven, CT) on the plantar surface of the injected paw, heated at a constant rate of 1.5 °C/s from 35 °C to a maximum of 53 °C. To avoid damage to the paw, the Peltier device was removed when the animal withdrew. Paw withdrawal was determined based on EMG activity.

Next cell responses to light were tested by placing a fiber-optic light source (Dolan-Jenner Fiber-Lite; Dolan-Jenner Industries, Buxborough, MA) 5 cm from the left eye to deliver diffused light at a range of intensities in ascending order (30, 140, 330, 900, and 6,000 lux). Each light trial lasted 30 s with each intensity repeated three times. Heat-evoked withdrawal threshold was determined in two final heat trials to confirm anesthetic stability (Merkel et al., 1963). At the conclusion of each experiment inflammation was confirmed visually and paws were measured with calibrated calipers applied at the widest point across the dorsal-plantar surface.

3.4.4 Histology

At the end of each experiment, the recording site was marked with an electrolytic lesion. Animals were euthanized by methohexital overdose and perfused transcardially with saline and 10% formalin. Brains were removed, and brainstems were sectioned on a Leica CM3050 S cryostat (60 µm sections). RVM lesion was photographed with an Optronics Microfire camera attached to an Olympus BX51 microscope. RVM was defined as the nucleus raphe magnus and adjacent reticular formation medial to the lateral boundary of the pyramids at the level of the facial nucleus (Paxinos et al., 2009).

3.4.5 Data Processing and Analysis

At the conclusion of each experiment, action potential waveforms were individually examined to verify correct waveform sorting. Paw withdrawal latency was defined as the average time from heat onset till paw withdrawal based on EMG activity, and averaged across all trials for each animal.

Ongoing activity was defined as the average firing rate during three 30-s periods, measured every 2.5 min during the 10-min unstimulated, baseline period prior to the first set of heat trials. Heat evoked reflex-related neuronal activity for ON-cells was defined as the total number of spikes in the burst and the duration of the burst, where a “burst” is defined as the first action potential after heat onset until the last action potential that precedes a 2-s quiet period. If a cell was active prior to heat onset, then the number of action potentials in the 3-s period around the paw withdrawal was used to define the number of spikes in the burst and burst duration was not defined for these cells. Heat-evoked reflex-related neuronal activity for OFF-cells was defined as the percent suppression quantified by the firing rate in the 3-s period around the paw withdrawal relative to the firing rate 30-s prior to heat onset and as the duration of the pause. A “pause” was considered to begin at the last spike after heat onset that was not followed within 2 s by another action potential and lasted until there were two action potentials within 2 s of each other. Percent pre-stimulus firing was also determined for NEUTRAL-cells based on the firing rate in the 3-s period around the paw withdrawal compared to the 10-s period preceding heat onset.

Overall a cell was considered to be “light-responsive” if it had a positive response at 6,000 lux. A positive response was one in which cell activity changed by at least 50% during light exposure relative to the 30-s period preceding light onset. In addition, for a response to be considered positive, a minimum of 10 action potentials during the light

stimulus was required of ON-cells, and a minimum of 10 action potentials prior to light stimulus was required of OFF-cells. The percentage of cells that responded within each light intensity was also determined, following the same criteria to define a response as positive. Lower intensities were used in this study as opposed to chapter 2. In chapter 2 it is likely that there was a floor effect of light intensity, because there was nearly maximal activation of ON-cells and suppression of OFF-cells even at the lowest intensity.

For light-responsive cells, stimulus-response curves for light-related firing activity were generated based on the average of three light trials at each intensity. For ON-cells, the total number of action potentials in the burst and duration of the burst were defined following similar criteria to define a “burst” as in heat trials. If an ON-cell was active prior to a light trial, then the total number of spikes during the light trial was used to define the number of spikes in the burst, and burst duration and latency were not quantified for the given trial. For OFF-cells, the percent suppression was defined as the firing rate during light exposure relative to the firing rate in the 30-s period preceding the light trial. The longest OFF-cell pause initiated during light exposure was also defined following similar criteria to define a “pause” as in heat trials. Latency to response was also determined as defined by the time from initiation of the trial to the start of an ON-cell “burst” or OFF-cell “pause”. Latency was not determined for trials were an OFF-cell was inactive or ON-cell active prior to light. For ON-cells, the average firing rate and peak firing rate during light exposure were also determined.

Some cell parameters were highly skewed based on distribution, so these parameters were log transformed to normalize data. Comparisons of ongoing activity and heat-evoked cell activity and paw withdrawal were made using a 2-factor ANOVA with sex and treatment as factors. Proportion of light-responsive cells was compared

between the sexes with Chi-squared test and stimulus-response for proportion of light-responsive cells was determined using Chi-squared test for trend. Area-under-the-curve was determined for light-evoked response magnitudes and compared using a 2-factor ANOVA with sex and inflammation as factors. If the overall ANOVA revealed significant differences, then Sidak's post-hoc test was used to compare between groups at each intensity. When ANOVA reveal no differences, groups were collapsed to determine stimulus-response relationships. A stimulus-response relationship was considered significant if a one-sample *t*-test determined average slopes were significantly different from zero. For all tests, $p<0.05$ was considered significant.

3.5 Results

3.5.1 Persistent inflammation following CFA injection does not produce thermal hypersensitivity or change the noxious stimulus-related responses of OFF- or ON-cells in female animals

Animals were treated with an injection of CFA or saline (control) in the hindpaw 5-6 days prior to recording. The injected paw of CFA-treated animals was significantly larger than that of saline-treated animals ($F(1,111)=506.9$, $p<0.0001$, $n=115$; M SAL: 6.71 +/- 0.45; M CFA: 8.90+/- 0.48 mm; F SAL: 6.06+/- 0.68; F CFA: 8.78 +/- 0.69 mm) on the day of recording. OFF- and ON-cells were sampled at random and defined based on response to noxious heat stimulation, and then tested for response to a range of light intensities. A total of 76 cells were recorded from 57 males, and 72 cells from 58 females (1-3 cells per animal). In both sexes sampled cells were distributed throughout RVM (Fig 11). Figure 12 shows examples of the noxious heat-related responses that were used to define RVM cells in male and female animals, illustrating that OFF-cells have a "pause" in ongoing activity and ON-cells have a "burst" of activity just prior to nocifensive paw withdrawal. NEUTRAL-cells were not tested in this study because prior work has shown

that none respond to light (Martenson et al., 2016, Chapter 2) and that responses to somatic stimuli are not altered during inflammation (Cleary et al., 2013).

The first goal of this project was to characterize the basic physiology of RVM cells in females with persistent inflammation. This was achieved by defining heat-evoked responses and ongoing cell activity in females, and comparing them to known responses in males. While, it was shown previously that males with persistent inflammation exhibit hyperalgesia to mechanical but not thermal stimuli, and that RVM neurons are sensitized to mechanical, but not thermal stimuli (Cleary et al., 2013, Pinto-Ribeiro et al., 2008, Almarestani et al., 2011), noxious heat is a convenient and reliable way to characterize RVM cells. We found that, as in males, persistent inflammation did not cause thermal hypersensivity in females, and that there was no difference in thermal sensitivity between the sexes, based on a lack of significant differences in noxious heat-evoked paw withdrawal latency (Fig 13). Additionally, there was no effect of inflammation on heat-evoked OFF-cell suppression, as defined by the percent firing during paw withdrawal relative to the period prior to heat onset (Fig 14a), or ON-cell burst, as defined by the total number of action potentials (Fig 14b), in either sex. There was also no effect of inflammation on the ongoing activity of either cell type based on the average firing rate during the baseline periods where no stimulation was applied to the animal (Fig 14c,d). These findings validate the defining features of RVM cells in females and agree with prior work in males (Cleary et al., 2013).

3.5.2 Light-evoked stimulus-response curves of RVM pain-inhibiting OFF-cell are shifted in animals with persistent inflammation

The second goal of this paper was to determine if light-evoked stimulus-response curves for RVM neurons are altered in a CFA model of persistent inflammation. Figure 15 shows example responses recorded simultaneously from a light-responsive OFF-cell

and ON-cell in a male CFA-treated rat during exposure to 30, 140, and 6,000 lux. As illustrated, light-responsive OFF-cells exhibit a characteristic “pause” in ongoing activity, while light-responsive ON-cells exhibit a characteristic “burst” in activity. A change in firing rate of at least 50% during exposure to light relative to the 30-s pre-stimulus activity was used to define a trial as a positive response. There was a significant response from some RVM cells even at 30 lux (Fig 15), the lowest intensity tested, indicating there may have been a floor effect of light intensity.

First, we compared the overall proportion of photoresponsive cells to determine if additional cells are recruited to the light-responsive population during persistent inflammation. There was no significant effect of treatment on overall proportion of photoresponsive OFF-cells (Fig 16a), based on the proportion of cells that were responsive to 6,000 lux, the highest intensity tested. Thus it does not appear that additional OFF-cells are recruited to the light-responsive subset of RVM cells during persistent inflammation. Next, we compared the proportion of cells responsive to lower light intensities to determine if the threshold to evoke a response was altered. We found that stimulus-response curves for the proportion of OFF-cells were left-shifted in animals with inflammation (Fig 16a). Further analysis revealed that in animals with persistent inflammation, there was a significant increase in the proportion of cells that responded to 140 lux, indicating that OFF-cell activity is inhibited by lower light intensities during inflammation.

Next, among light-responsive RVM cells, we established stimulus-response curves for light-evoked changes in cell firing to determine if persistent inflammation alters the magnitude of light-evoked activity. Inflammation had a significant effect on light-evoked OFF-cell suppression such that stimulus-response curves for percent firing during light relative to the 30-s prior to light onset were left-shifted (Fig 16b). Follow up testing

revealed 140 lux induced significantly more OFF-cell suppression in inflamed animals compared to control animals. However, inflammation did not have an effect on the duration of longest pause during light exposure or time from light onset to pause (latency to pause) (Table 2).

3.5.3 RVM pain-facilitating ON-cell response to light is not altered in animals with persistent inflammation

Stimulus-response curves for RVM ON-cells were not altered by persistent inflammation. We found that there was no significant effect of inflammation on the proportion of ON-cells that responded to light at any intensity (Fig 17a). ON-cell burst during light, as defined by the total number of spikes in the burst (Fig 17b), by the duration of burst, by the peak firing rate during light, or by latency to a burst (Table 2) was not significantly different in inflamed compared to control animals.

In summary, these results show that during persistent inflammation the light-evoked response threshold of OFF-cells is decreased in both sexes and dim levels of light evoke greater OFF-cell suppression, but that ON-cell response to light is unaltered. Additionally, we showed previously that RVM cells in naïve male and female animals have similar light-evoked stimulus-response curves (Chapter 2). In CFA-treated animals, sex did not have a significant effect on light-evoked responses of OFF- or ON-cells (Figures 16 and 17, respectively), showing that persistent inflammation does not have different effects in the two sexes. As with prior work in naïve animals (Chapter 2), RVM cell responses to light were also found to be graded in the present study.

3.6 Discussion

The primary goal of this study was to determine the effects of persistent inflammation on RVM cell light-evoked stimulus-response curves in male and female animals.

However, we first looked at ongoing and heat-evoked RVM cell activity to validate that, as in male animals, there is no effect of persistent inflammation on response to noxious heat in females. We found that inflammation did not have an effect on ongoing or heat-evoked responses in female animals. This is consistent with findings in males showing that with persistent inflammation RVM cells do not have altered responses to thermal stimuli, but in contrast develop more robust responses to mechanical stimuli (Cleary et al., 2013, Montagne-Clavel et al., 1994, Pinto-Ribeiro et al., 2008). Since RVM ON- and OFF-cells are defined by their responses to noxious heat, it was important to validate baseline responses of RVM cells in females.

These observations on RVM cell activity are consistent with our finding that CFA-treatment did not have an effect on the thermal withdrawal threshold 5-6 days post-CFA, and are also in agreement with previous findings that, in male animals, thermal hyperalgesia begins to resolve within the first 24 hours (Cleary et al., 2013, Pinto-Ribeiro et al., 2008, Guan et al., 2003, Ren et al., 1996, Wei et al., 1999, Okun et al., 2011, Almarestani et al., 2011). Although thermal hyperalgesia peaks at 24 hours, some, but not all, studies in awake behaving animals find it to be present for longer (Wang et al., 2006, Ren et al., 1992, Wei et al., 1999). However, many studies in awake behaving animals use radiant heat with a logarithmic rise in temperature, whereas we used contact heat with a slow linear ramp. The former has been shown to be more likely to detect thermal hyperalgesia (McMullan et al., 2004, Yeomans et al., 1996, Baba et al., 1999). Therefore, it is possible that we missed thermal hyperalgesia and any sex-related differences in thermal hyperalgesia. However, there are conflicting results regarding sex differences in CFA-induced hyperalgesia (Wang et al., 2006, Loyd et al., 2008, Craft et al., 2013, Cook et al., 2005, Cook et al., 2006, Bradshaw et al., 2000). Results are influenced by factors such as time course, nociceptive testing assay, dependent variable

measured, and animal strain (Cook et al., 2006, Mogil et al., 2000, Cook et al., 2005). Overall, differences in both acute nociceptive sensitivity and in hyperalgesia during persistent inflammation are likely nonexistent or minor (Mogil et al., 2005).

Despite similar behavioral endpoints, there are qualitative differences in pain-modulation circuitry between the sexes that could underlie observed discrepancies in prevalence and presentation of chronic pain disorders. For example, periaqueductal gray (PAG) input to RVM is critical to pain-modulation, and during CFA-induced inflammation there is greater PAG-RVM output activated in males than in females (Loyd et al., 2008). Importantly, this study also found that there are no differences in CFA-induced hyperalgesia between the two sexes, making the behavioral relevance of greater PAG-RVM output activation by inflammation unclear. However, our observation that RVM activity is similar in males and females, together with findings that there are no differences in behavioral hyperalgesia following CFA (Wang et al., 2006, Loyd et al., 2008, Cook et al., 2006, Craft et al., 2013), indicates that molecular and anatomical differences do not necessarily lead to differences in output of the pain-modulation system or behavioral endpoint.

After showing that RVM ongoing activity and heat-evoked responses in females with persistent inflammation are similar to those in males, we tested the effects of inflammation on RVM responses to light, a non-noxious stimulus. As with prior work in uninjured animals (chapter 2), we found ON- and OFF-cell responses to light were similar between the sexes. Furthermore, inflammation did not alter the overall proportion of cells responsive to bright light or the light-evoked changes in firing during exposure to bright light. This indicates that inflammation neither recruits additional cells to the photoresponsive population nor changes the maximum light-evoked response.

However, within the light-responsive population, inflammation led to an increase in the proportion of OFF-cells responding to light at 140 lux and OFF-cell firing in inflamed animals was more suppressed by 140 lux. This parallels the effect of persistent inflammation on response to mechanical stimulation (Cleary et al., 2013), in that there are no differences observed at higher intensities or in the maximum evoked-response but the response to lower intensities is left-shifted. Additionally, within the light-responsive population, there were no effects of inflammation on ON-cell response to lower light intensities. This latter finding is in contrast to the effects of persistent inflammation on mechanically-evoked responses, in which the ON-cell response curve is also left-shifted (Cleary et al., 2013, Montagne-Clavel et al., 1994). Therefore, the brightness threshold at which descending inhibition is removed is lowered based on the shift in the OFF-cell stimulus-response curve, while light-evoked descending facilitation may not be altered.

Importantly, prior work showed response to mechanical stimulation was only altered during stimulation of the inflamed paw and not during stimulation of the contralateral paw (Cleary et al., 2013). Therefore, enhanced response to somatosensory input may only be detected when ascending input from the injury is active. Although sensitization to visual light was limited to OFF-cells, enhanced response in the absence of activating ascending input from the injury site, supports our suspicion that light engages RVM through a pathway separate from the somatosensory system (Martenson et al., 2016, chapter 2). We suspect that the effects of light on RVM activity may be through a “top-down” mechanism by engagement of higher relays that ultimately effect RVM output, because RVM response to light appears to be independent of the primary sensory system (Martenson et al., 2016). Rather, prior work showed that RVM response to light

is dependent on the olivary pretectal nucleus (OPt), which mediates the pupillary light-reflex and contains light-responsive neurons (Clarke et al., 1980).

Another important difference between somatic- and light-evoked responses of RVM cells is that in both naïve and inflamed animals, responses to light are graded, whereas response to somatic stimulation is all-or-none in naïve animals and graded in animals with persistent inflammation (Cleary et al., 2013). The changes observed in RVM cell responses to mechanical stimuli are in the non-noxious range where cells in naïve animals do not respond. In contrast, even at the lowest light intensity (30 lux) there was a response from a subset of cells within all groups. Therefore, response to light is qualitatively different from response to somatic stimulation in the naïve state so it is possible that this limits our ability to obverse plasticity in the light response.

Previously it was shown that in naïve animals, exposure to 18,000 lux slightly lowers thermal withdrawal threshold (Martenson et al., 2016). However, the current study and previous findings (chapter 2) show that RVM cells respond to light intensities much lower than 18,000 lux, with over 50% of cells responding to 140 lux. Therefore, effects of other light intensities on behavioral responses will need to be determined in future work. While the effect of inflammation on OFF-cells was small, the amount of light required to observe this effect is equivalent to a very dimly lit room, so, in injury, very low levels of light could diminish descending inhibition and have behavioral consequences. Thus, future behavioral experiments in injured animals will be especially important to determine if light concurrent with nociceptive stimuli can act in an additive or even multiplicative fashion since RVM neurons are also sensitized to mechanical stimuli. Thus, engagement of RVM by light could further tip the balance in an already sensitized pain-modulation system causing normal somatosensory afferent traffic to be perceived as aversive or even painful, resulting in general somatic discomfort and widespread pain.

In this project, we chose the hindpaw model because, while we believe engagement of light is not dependent on the trigeminal sensory system, models in which the head and orofacial tissues are sensitized could confound potential trigeminal engagement. While hindpaw inflammation results in modest plasticity in OFF-cell response to light, future work will be needed to determine if the light-evoked stimulus-response curve is shifted in other pain models, such as migraine or temporomandibular joint disorder. These disorders may be accompanied by a greater degree of central sensitization, as indicated by hypersensitivity at locations remote from the injury Edelmayer et al., 2009, Chai et al., 2012, Ambalavanar et al., 2006), so they may exhibit a more robust effect.

In conclusion, this project shows that the threshold for light-evoked RVM cell response is equivalent to a dimly lit room and that inflammation further decreases the threshold at which light-stimulation reduces descending inhibition.

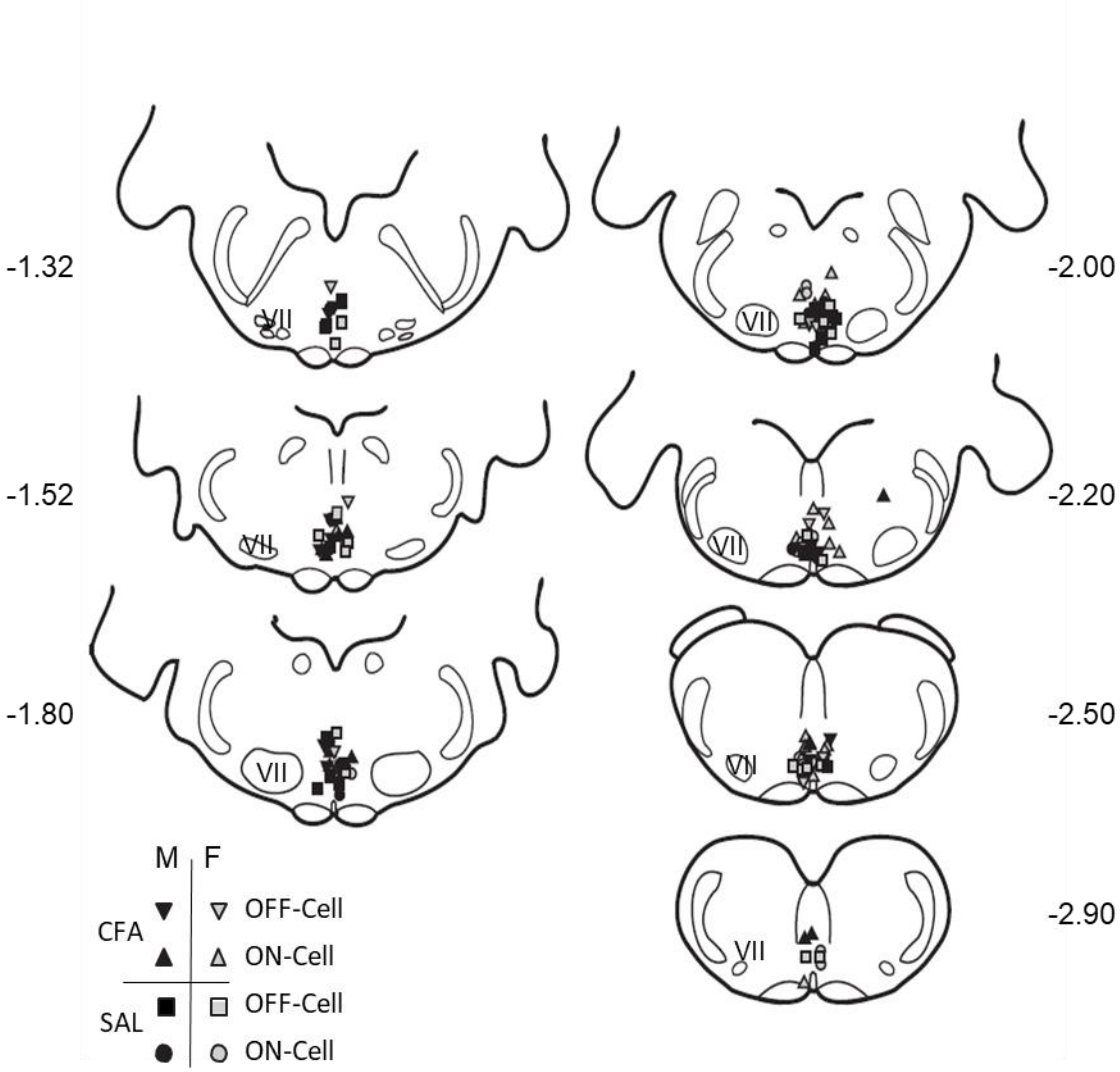

Figure 11 Recording locations within the RVM

OFF- and ON-cells were distributed between -1.32 mm and -2.90 mm relative to the interaural line, with the majority of the cells recorded between -1.80 mm and -2.5 mm. There were 23 ON- and 17 OFF-cells recorded from 28 CFA-treated males, 17 ON- and 19 OFF-cells recorded from 29 control males, 20 ON- and 14 OFF-cells recorded from 30 CFA-treated females, and 18 ON- and 20 OFF-cells recorded from 28 control females. VII: facial nucleus.

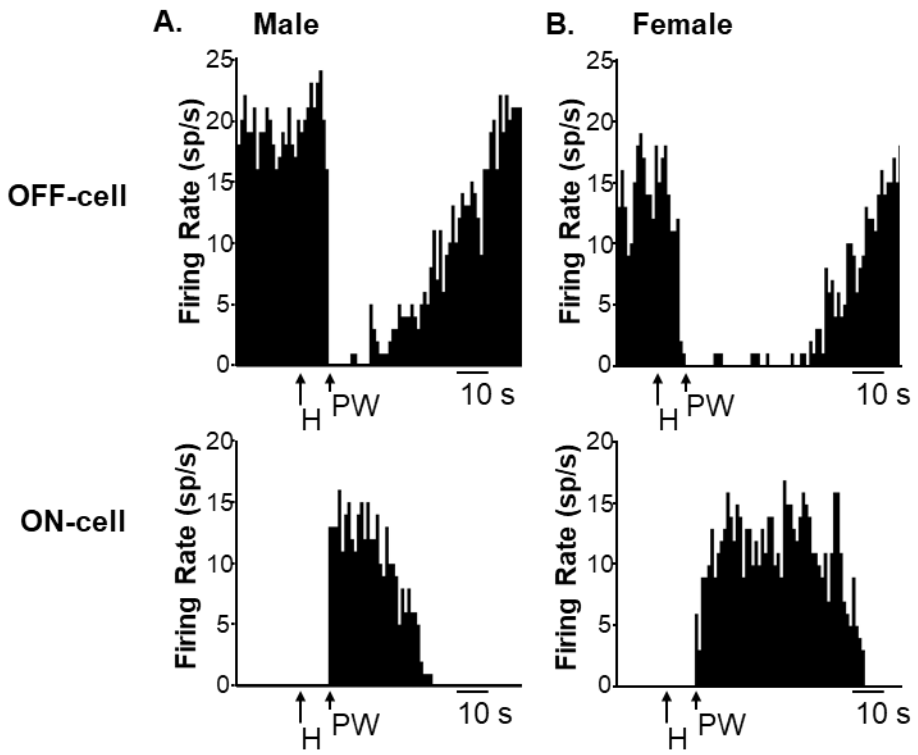

Figure 12 Representative heat-evoked responses of OFF-cells (upper) and ON-cells (lower)

Ratemeter records (1-s bins) show heat-evoked cell responses recorded from RVM in male (left) and female (right) rats, with heat onset (H) and paw withdrawal (PW). OFF-cells have a pause in ongoing activity while ON-cells have a burst of activity just after heat onset and prior to paw withdrawal. OFF-cell response can be quantified based on the percent suppression relative to the ongoing firing rate prior to stimulus onset and ON-cell response can be defined based on the total number of action potentials in the burst. Additionally, the duration of a response and latency to a response can be quantified.

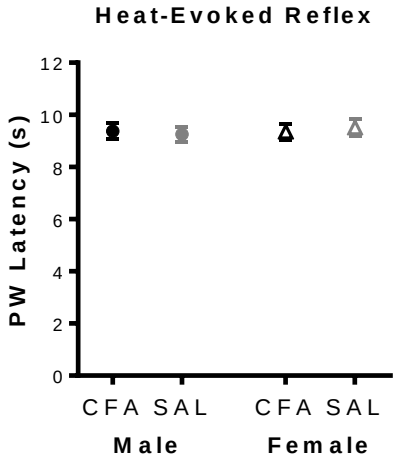

Figure 13 Thermal withdrawal latency

There was no significant effect of treatment ($F(1,111)=0.016$, $p=0.90$), sex ($F(1,111)=0.60$, $p=0.44$), or sex X treatment interaction ($F(1,111)=0.94$, $p=0.34$) on noxious heat-evoked paw withdrawal latency. Data are displayed as mean +/- 95% CI, n=28-30 cells/group.

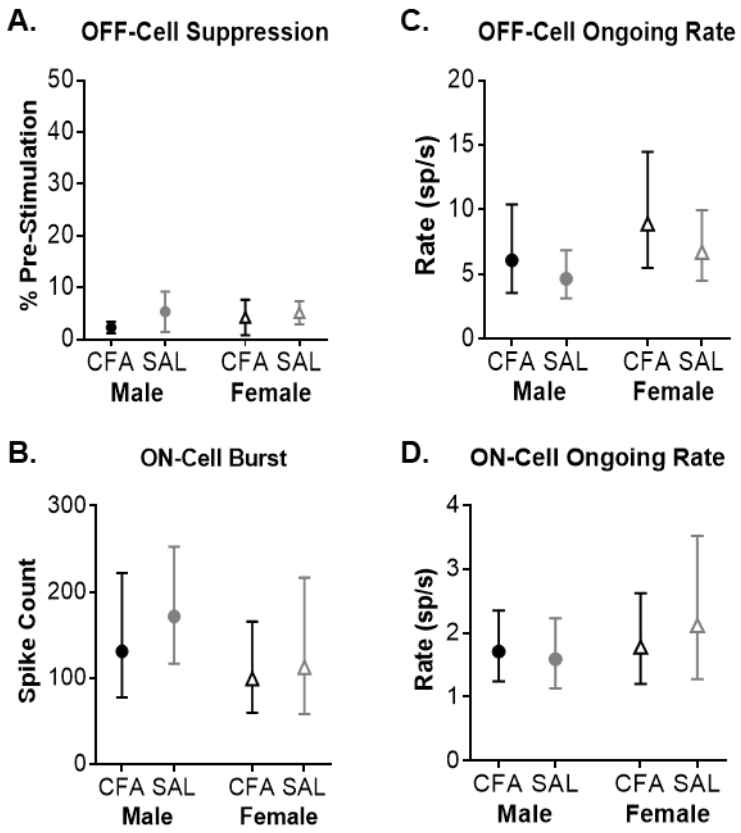

Figure 14 Ongoing activity and heat-evoked responses of RVM OFF-and ON-cells

A. *OFF-cell suppression.* OFF-cell response, as defined by the percent suppression of firing during paw withdrawal relative to the firing prior to heat onset, revealed that there was no significant effect of treatment ($F(1,66)=2.08$, $p=0.15$), sex ($F(1,66)=0.41$, $p=0.53$), or sex X treatment interaction ($F(1,66)=0.60$, $p=0.44$). Data are displayed as mean +/- 95% CI, n=14-20 cells/group.

B. *ON-cell burst.* ON-cell response, as defined by the total number of action potentials in the “burst”, revealed that there was no significant effect of treatment ($F(1,74)=0.59$, $p=0.45$), sex ($F(1,74)=1.88$, $p=0.17$), or sex X treatment interaction ($F(1,74)=0.085$, $p=0.77$). Data are displayed as geometric mean +/- 95% CI, n=17-23 cells/group.

C. *OFF-cell ongoing activity.* There was no significant effect of treatment ($F(1,66)=2.04$, $p=0.16$), sex ($F(1,66)=2.98$, $p=0.089$), or sex X treatment interaction ($F(1,66)=0.000094$,

p=0.99) on OFF-cell ongoing firing rate based on average firing during the unstimulated, baseline period. Data are displayed as geometric mean +/- 95% CI, n=14-20 cells/group.

D. *ON-cell ongoing activity.* There was no significant effect of treatment ($F(1, 74)=0.079$, $p=0.78$), sex ($F(1,74)=0.77$, $p=0.38$), or sex X treatment interaction ($F(1,74)=0.44$, $p=0.51$) on ON-cell ongoing firing rate based on average firing during the unstimulated, baseline period. Data are displayed as geometric mean +/- 95% CI, n=17-23 cells/group.

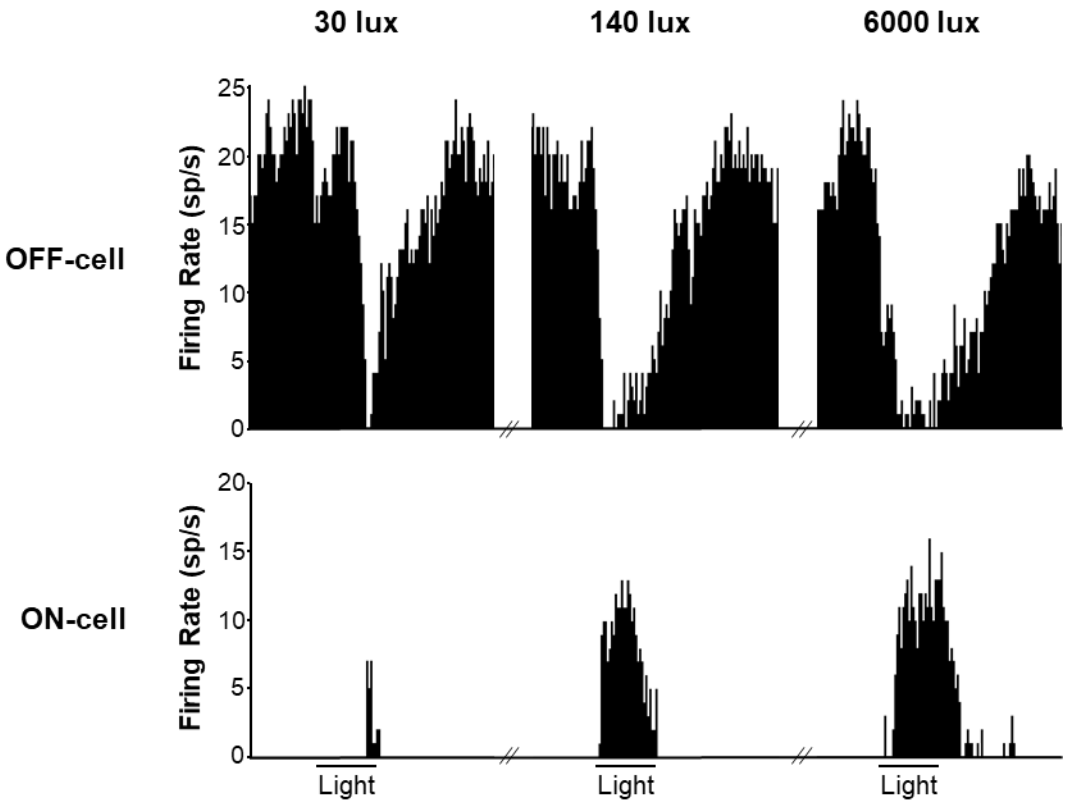

Figure 15 Representative RVM OFF-cell (top) and ON-cell (bottom) responses to visual light

Ratemeter records (1-s bins) from an OFF- and ON-cell recorded simultaneously in RVM in a male CFA-treated animal, with 30-s light stimulus denoted below the traces. Examples show clear light-evoked suppression of OFF-cell activity and activation of ON-cell during exposure to light at 30, 140, and 6000 lux.

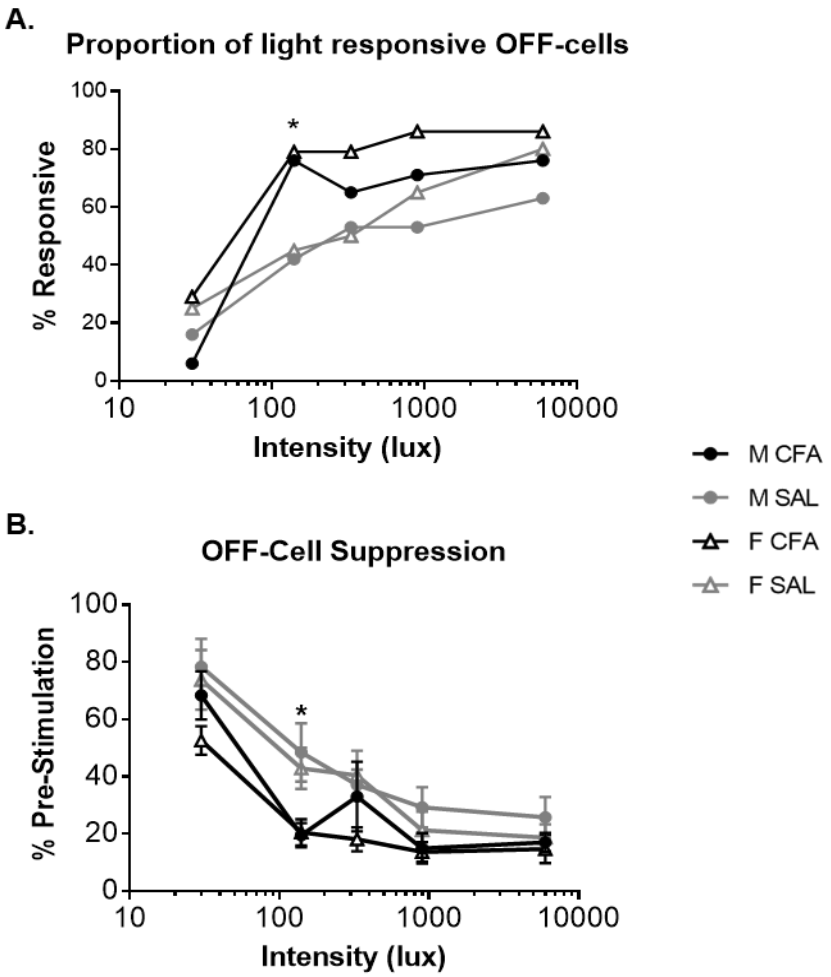

Figure 16 RVM OFF-cell response to light is enhanced during persistent inflammation

A. Percent light-responsive OFF-cells. There was no significant difference between groups in the overall proportion of light responsive OFF-cells based on the proportion of cells that responded to 6,000 lux ($X^2(3)=2.60$, p=0.46). However, there was a significant effect of inflammation on the proportion of OFF-cells that responded to 140 lux (Fisher's exact, p=0.0043). Additionally, there was a significant stimulus-response effect of light intensity on proportion of responsive cells (chi-squared test for trend, SAL: ($X^2(1)=21.71$, p<0.0001; CFA: ($X^2(1)=20.19$, p<0.0001). Data are displayed as the percent of cells with

a positive response at each light intensity, as defined by a change in firing rate of at least 50% compared to pre-stimulus firing, n=14-20 cells/group.

B. Light-evoked OFF-cell suppression. There was a significant effect of treatment ($F(1,49)=6.64$, $p=0.013$), but no significant effect of sex ($F(1,49)=0.99$, $p=0.32$), or group X intensity interaction ($F(1,49)=0.044$, $p=0.83$) on OFF-cell suppression, as defined by the percent firing during light exposure compared to firing in the 30-s prior to light. Follow-up testing revealed a significant effect of treatment at 140 lux (Sidak's multiple comparisons, $p=0.0027$). Additionally, there was a significant stimulus-response effect of light intensity on proportion of responsive cells (CFA: $t(24)=7.96$, $p<0.0001$; SAL: $t(27)=8.70$, $p<0.0001$). Data are displayed as mean +/- SEM, n=12-16 cells/group.

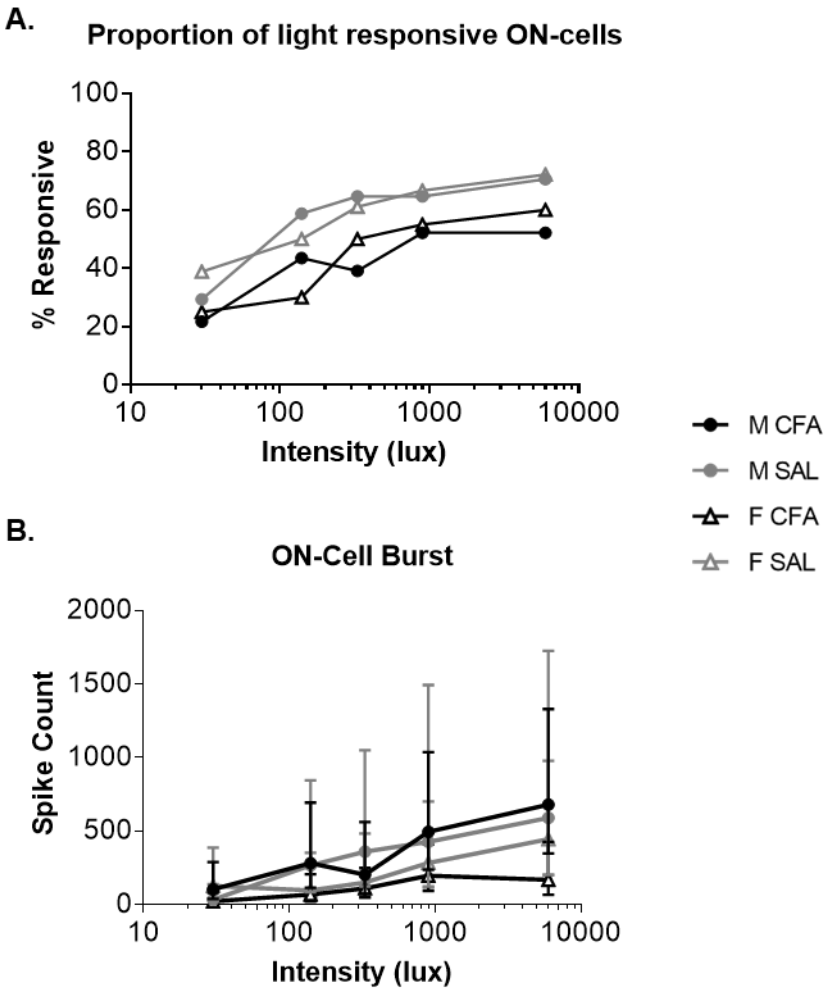

Figure 17 RVM ON-cell response to light is not altered in persistent inflammation

A. Percent light-responsive ON-cells. There was no significant difference between groups in the overall proportion of light-responsive ON-cells based on the proportion of cells that responded to 6,000 lux ($X^2(3)=2.31$, p=0.51). There was a significant stimulus-response effect of light intensity on proportion of responsive cells (chi-squared test for trend, $X^2(1)=21.67$, p<0.0001). Data are displayed as the percent of cells with a positive response at each light intensity, as defined by a change in firing rate of at least 50% compared to pre-stimulus firing, n=17-23 cells/group.

B. Total spikes in light-evoked ON-cell burst. There was no significant effect of treatment ($F(1,45)=0.47$, $p=0.50$), sex ($F(1, 45)=2.89$, $p=0.096$), or sex X treatment interaction ($F(1,45)=1.28$, $p=0.26$) on total spikes in the ON-cell “burst”. Light intensity had a significant stimulus-response effect on ON-cell burst ($t(48)=9.18$, $p<0.0001$). Data are displayed as geometric mean +/- 95% CI, n=12-13 cells/group.

Cell Type	Parameter	Effect of sex?	Effect of treatment?	Stimulus Response Effect?
OFF	Longest pause duration	No (F(1,49)=2.47, p=0.12)	No (F(1,49)=1.54, p=0.22)	Yes (t(52)=10.45, p<0.0001)
OFF	Latency to pause	No (F(1,49)=1.08, p=0.30)	No (F(1,49)=1.83, p=0.18)	Yes (t(52)=6.70, p<0.0001)
ON	Firing rate during light	No (F(1,45)=0.21, p=0.65)	No (F(1,45)=1.19, p=0.28)	Yes (t(48)=8.76, p<0.0001)
ON	Peak firing rate during light	No (F(1,45)=0.048, p=0.83)	No (F(1,45)=0.17, p=0.69)	Yes (t(48)=7.47, p<0.0001)
ON	Light evoked burst duration	No (F(1,44)=0.02, p=0.88)	No (F(1,44)=3.70, p=0.061)	Yes (t(47)=9.37, p<0.0001)
ON	Latency to burst	No (F(1,44)=0.0069, p=0.93)	No (F(1,44)=1.87, p=0.18)	Yes (t(47)=3.69, p=0.0006)

Table 2 Additional ON- and OFF-cell light-evoked response parameters

Other parameters that can be used to quantify ON-cell response to light include the firing rate during light exposure, peak firing rate during light, duration of burst (from time of first light-evoked spike to the first 2-sec quiet period), and latency to burst (time from light onset to initiation of the burst). Other parameters that can be used to quantify OFF-cell response to light include the longest pause duration and latency to pause (time from light onset to longest pause). These parameters demonstrate that there are no significant differences between the sexes and no significant effect of treatment among the light-responsive subset of cells. They also further support that there is a stimulus-dependent effect of light intensity on cell response.

Note: “Light evoked burst duration” and “Latency to burst” were not calculated for cells that were active at the initiation of a light trial, so some values are missing for these parameters.

CHAPTER 4

DISCUSSION

4.1 Key findings

- These studies establish a model for using female rats in electrophysiology studies utilizing a lightly anesthetized preparation.
- RVM cell responses during noxious heat-evoked withdrawal and light exposure are similar in male and female rats, showing that output from the pain-modulating system is similar in the two sexes.
- Persistent inflammation does not produce thermal hyperalgesia or change the heat-evoked responses of RVM cells in females, similar to previous findings in males.
- Very dim levels of visual light can activate ON-cells and suppress OFF-cells. Furthermore, light-evoked responses of ON- and OFF-cells are graded with stimulus intensity in both the naïve and inflamed state. This is in contrast to noxious somatic stimulus-evoked responses, which are all-or-nothing.
- In both sexes, persistent inflammation enhances the responsiveness of OFF-cells to dim light, but does not change the responsiveness of ON-cells.

4.2 Overview

The RVM is the output node of a major pain-modulation circuit and exerts bidirectional control of nociceptive transmission via projections to the dorsal horn and trigeminal nucleus. There are two classes of cells in RVM that are responsible for this modulation, pain-facilitating ON-cells and pain-inhibiting OFF-cells. These two classes of cells are defined based on their changes in firing during nociceptive withdrawal; ON-cells have a burst in activity while OFF-cells pause any ongoing activity. While the physiology of RVM has been well-characterized in male animals, there is very little known about the behavior of these cells in female animals. There are anatomical and molecular

differences in pain-modulation circuitry between males and females, but it is unknown if these differences result in differential output from RVM. Therefore, one goal of this thesis was to determine whether the physiological responses of RVM neurons are similar in males and females.

Interestingly, these pain-modulating neurons respond to visual light. Therefore, engagement of RVM by light could modulate nociception. A second goal of this thesis was to develop stimulus-response curves to further characterize RVM response to light. Additionally, during persistent inflammation or prolonged injury, RVM neurons are sensitized and develop enhanced responses to somatic stimulation. While the effects of inflammation on the RVM response to light are unknown, a more robust response from photoresponsive cells or recruitment of cells to the photoresponsive population could contribute to photophobia. Thus, a third goal of this thesis was to determine the effects of persistent inflammation on RVM cell light-evoked stimulus-response curves.

4.2.1 The output of the pain modulation system is comparable in males and females

Historically, female animals have been excluded from basic pain research. There are many reasons researchers may have chosen to use males over females, including concerns that female animals would increase variability in data or assumptions that findings from studies in male animals could be extrapolated to females. These ideas are now thought to be unfounded, and females have been shown to be no more variable than males (Mogil et al., 2005, Becker et al., 2016, Beery et al., 2011, Prendergast et al., 2014). However, there are demonstrated sexual dimorphisms in pain circuitry, and thus findings from males should not simply be applied to females. In this thesis, I consider the possibility that sexual dimorphisms exist in RVM cell activity, the physiological output of the pain-modulation system.

In chapter 2, I demonstrated that there are no differences in RVM cell ongoing activity and heat-evoked responses in males and females under basal conditions. The

ongoing activity of RVM cells sets the threshold for behavioral withdrawal to noxious stimuli (Heinricher et al., 1989). Therefore, my finding that the ongoing firing rate is similar between the sexes is consistent with my behavioral findings, and those of others, showing that there are no sex-related differences in nociceptive sensitivity (Loyd et al., 2008, Wang et al., 2006, Boyer et al., 1998, Bradshaw et al., 2000, Terner et al., 2005, Bobeck et al., 2009, Tershner et al., 2000, Doyle et al., 2018, Doyle et al., 2018, Cook et al., 2006). During ON-cell burst and OFF-cell pause, there is a state of hypersensitivity, meaning that subsequent stimuli can evoke a response at a lower threshold (Ramirez et al., 1989). Thus, similar magnitudes of ON-cell burst and OFF-cell pause in males and females, also supports females are no more likely than males to be sensitized by repeated noxious stimulation (Lomas et al., 2005).

Chapter 2 also showed that light-evoked responses are not different in the two sexes. In a subset of RVM cells, light can inhibit pain-inhibiting OFF-cells and activate pain-facilitating ON-cells. Thus light could lower nociceptive thresholds by modulating the ongoing activity of RVM cells. If more cells were responsive to light or if the light-responsive subset of cells had a more robust response in one sex than the other, there would likely be behavioral implications. For example, one possibility is that light could have different effects on nociceptive sensitivity in the sexes, and light conditions might be another factor contributing to the variability in the results of sex difference studies on nociceptive sensitivity. However, my results show it is unlikely that light is a factor influencing sex differences in nociceptive sensitivity.

Many chronic pain conditions are more prevalent in females (Fillingim et al., 2009, Buse et al., 2013, Lipton et al., 2001a, Bolay et al., 2015, Unruh, 1996) and animal studies show there are differences in PAG output to RVM during persistent inflammation (Loyd et al., 2008, Loyd et al., 2006). Thus, sexual dimorphisms in pain-modulation circuitry during inflammation could explain why chronic pain disorders are more

prevalent in females. Therefore, while there were no differences found in the basal state, it was plausible that the effects of inflammation would be different between the two sexes. However, in chapter 3, I found OFF-cell response to light was enhanced in both sexes, while ongoing activity and heat-evoked responses were not altered during persistent inflammation. In summary, there were no significant differences in the effect of inflammation on ON- and OFF-cell activity in RVM in male and female animals based on ongoing activity, or heat- and light-evoked responses.

These findings indicate that it is unlikely that there are sex differences in RVM output during persistent pain that contribute to sex-related disparities in chronic pain conditions. A caveat to this finding is that the only somatic stimulus utilized was noxious heat. Previous work has shown that in lightly anesthetized male animals, thermal hyperalgesia begins to resolve after the first 24 hours, and heat-evoked responses are not altered during persistent inflammation (Cleary et al., 2013, Guan et al., 2003, Ren et al., 1996, Wei et al., 1999, Okun et al., 2011). Even so, it was important to validate that, during persistent inflammation, females do not display thermal hyperalgesia or altered RVM cell responses to heat, as this would indicate there are likely different pain-transmission or modulation mechanisms at play. Testing for mechanically-evoked RVM cell response, which is known to be enhanced in males during persistent inflammation (Cleary et al., 2013), is needed to be able to draw a full conclusion about the effects of inflammation on RVM cell activity in females.

Interestingly, it was previously reported that there are more PAG-RVM projection neurons activated during CFA-induced inflammation in males than in females (Loyd et al., 2006). Importantly, that study also found that there are no differences in CFA-induced hyperalgesia between the two sexes, making the behavioral relevance of greater PAG-RVM output activation by inflammation unclear. However, my findings imply that this difference in PAG activation does not result in an overall difference in the

physiological output of the system, as measured by RVM cell activity, and behavior. Since RVM activity helps set the nociceptive “tone”, these findings explain why there are no sex-related differences in hyperalgesia in this model.

4.2.2 Light as a “top-down” input to RVM

How light reaches the RVM is still being elucidated. However, our lab showed previously that light-evoked responses are independent of primary sensory pathways (Martenson et al., 2016). Thus it is unlikely that light is simply acting as a noxious stimulus, and more probable that light acts as a “top-down” input to RVM (Fig 18). My findings support this by showing that very dim levels of light can evoke a response from RVM. These dim levels of light (140-330 lux) are much lower than the threshold to evoke a response from the trigeminal system (5,000 lux) (Okamoto et al., 2010).

My findings further support that light and somatic stimuli invoke separate pathways based on the qualitatively different RVM cell responses to light in comparison to somatic stimulation, in that response to light, even under basal conditions, is graded with stimulus intensity. While, nociceptive input is the primary drive that evokes the characteristic ON-cell burst and OFF-cell pause, forming a positive feedback loop that promotes responses to subsequent, potentially damaging stimuli (Heinricher et al., 1989, Ramirez et al., 1989), this feedback loop is modulated by input from higher structures. This input can shift the balance between pain-facilitation and pain-inhibition depending on biological needs (Martenson et al., 2009, Foo et al., 2005, Ren et al., 2002, Roeder et al., 2016, Calejesan et al., 2000, McGaraughty et al., 2002, Wagner et al., 2013, Sandkühler et al., 1984, Basbaum et al., 1984). My findings imply that light may act similarly to emotional and cognitive factors to influence the “tone” of the pain-modulation system. Light acting as a “top-down” input is a likely mechanism to explain lowered nociceptive thresholds in rats during light exposure (Martenson et al., 2016).

One structure that might be an important relay for light input to RVM is the olivary pretectal nucleus (OPt). OPt is involved in the pupillary light-reflex and contains a subset of neurons that are light-responsive (Clarke et al., 1980, Hattar et al., 2002) due to afferent projections from intrinsically photosensitive retinal ganglion cells (ipRGC's). Importantly, activity of ipRGC's is implicated in photophobia (Noseda et al., 2010, Johnson et al., 2010, Semo et al., 2010). Previously, our lab demonstrated that inactivating OPt blocks light-evoked responses in RVM (Martenson et al., 2016). Furthermore, OPt response to light is graded with light intensity (Clarke et al., 1985, Clarke et al., 1981), and OPt projects to the PAG (Klooster et al., 1995a, Klooster et al., 1995b). One possibility is that OPt is a relay for light-related input to engage RVM in a graded fashion. More work will be needed though to determine exactly how signals from OPt reach RVM.

4.2.3 Persistent inflammation enhances the responsiveness of OFF-cells to light

During persistent inflammation, RVM output is shifted to a net inhibitory state, which is likely driven by output from OFF-cells that suppress nociceptive transmission through projections to the dorsal horn (Cleary et al., 2013, De Felice et al., 2011, Gonçalves et al., 2007, Wei et al., 1999). In chapter 3, I showed that persistent hindpaw inflammation lowers the threshold at which RVM OFF-cells respond to light, and that OFF-cells are more greatly suppressed by dim light compared to uninjured controls. This change in threshold and sensitized response of OFF-cells could mediate behavioral hypersensitivity since lowered OFF-cell activity decreases nociceptive thresholds (Heinricher et al., 1989, Ramirez et al., 1989), and an individual RVM neuron can have diffuse projections, terminating in multiple levels of the dorsal horn, as well as the trigeminal nucleus (Lovick et al., 1983, Huisman et al., 1981, Basbaum et al., 1976). Further, plasticity in the pain-modulation system is important in pathological pain (Cleary et al., 2013, Porreca et al., 2001). Thus, light-evoked modulation of RVM descending

control could have widespread effects on pain transmission, and enhanced OFF-cell response to light could contribute to symptoms of photophobia.

4.3 Technical considerations

4.3.1 Anesthesia

All experiments were performed in lightly anesthetized animals. While anesthesia is a potential confounding factor, the use of spinal reflex as a measure of nociception has been well established in descending modulation studies. Moreover, RVM neurons with similar physiological characteristics have been studied in barbiturate-, ketamine- and halothane-anesthetized rats as well as decerebrate-unanesthetized and awake rats (McGaraughty et al., 1993b, McGaraughty et al., 1993a, Morgan et al., 1992, Clarke et al., 1994, Foo et al., 2005, Leung et al., 1999, Roeder et al., 2016, Calejesan et al., 2000, McGaraughty et al., 2002, Basbaum et al., 1984). Additionally pharmacological manipulations of RVM in anesthetized animals have outcomes similar to those in unanesthetized animals (Wagner et al., 2013, Sandkühler et al., 1984, Hurley et al., 2000, Smith et al., 1997, Kovelowski et al., 2000, Edelmayer et al., 2009, Porreca et al., 2001). Therefore the relationship between RVM descending modulation and nociceptive transmission is likely preserved in the anesthetized model.

Previously, female rats were not used in electrophysiology studies in our lab, and their use has been very limited in general. Thus anesthesia has an additional potential confound, because maintaining a stable and equivalent anesthetic plane in both sexes is critical to recording studies in RVM. Because male and female rats were age-matched, there was a significant difference in body size between the sexes, and thus different anesthetic rates were needed. Furthermore, male rats have more visceral fat than females, which can affect anesthetic metabolism (Leshner et al., 1973, Morimoto et al., 2003), so anesthetic requirements could not simply be scaled down by body weight. Therefore, anesthetic rates that induced a similar anesthetic depth in males and females

based on muscular tone and reflex withdrawal were established (Merkel et al., 1963). These parameters are highly correlated with physiological parameters that indicate overall anesthetic depth, and are commonly used as indicators of anesthetic depth, so are a valid method to establish an anesthetic plane in females that would be comparable to that established for males (Arras et al., 2001, Gustafsson et al., 1996, von Dincklage et al., 2010, Shafer et al., 2008, Terayama et al., 2000, Guan et al., 2002).

4.3.2 Consideration of estrous cycle effects

There has been speculation that the lack of observed sex differences in animal studies of nociceptive sensitivity is due to not controlling for estrous cycle phase, and that grouping females from all phases masks the effect of hormones (Craft, 2003, Greenspan et al., 2007). If this theory is correct, it implies that there are specific stages of the estrous cycle in which females are more sensitive. However acute nociceptive testing shows that there is no more variability within females than males, meaning it is unlikely that there is a "highly sensitive" phase of the cycle that is being hidden by "less sensitive" phases (Mogil et al., 2005, Becker et al., 2016, Beery et al., 2011, Prendergast et al., 2014). Furthermore, data from studies comparing nociceptive sensitivity in different phases of the cycle have inconsistent results, and often show no differences between the phases (Berkley et al., 2006, Mogil et al., 2010, Loyd et al., 2008, Wang et al., 2006, Ji et al., 2006, Bradshaw et al., 2000, Terner et al., 2005). One reason for the variability in results is that there are multiple ways to classify and compare estrous cycle status, and it could also be that changes or fluctuations in hormone levels are the mediating factor (Mogil et al., 2000). In any case, there are major differences in the estrous cycle of a rat and human, so effects of cycle in animal work cannot simply be extrapolated to explain the human condition (Greenspan et al., 2007).

Despite a lack of sex differences in nociceptive sensitivity, anatomical and molecular differences in pain-modulating circuitry have been documented. Therefore, it is possible

that there are circuit-based sexual dimorphisms associated with the estrous cycle. Nevertheless, studies on the effectiveness of morphine, which acts on descending pain-modulation circuitry, report differences in multiple directions (Loyd et al., 2008, Terner et al., 2005, Mogil et al., 2000, Stoffel et al., 2003). This again suggests that it is unlikely that there are clear differences in pain-modulation across the different phases.

Based on the lack of consistency in current literature and confounding factors, powering a study to compare between all phases of the estrous cycle is likely unwarranted unless initial results indicate there may be an effect of cycle (Mogil, 2016, Becker et al., 2005, Greenspan et al., 2007). Thus, in this thesis, estrous cycle for each rat was recorded at the end of each experiment by vaginal swab, and classified as either proestrus, estrus, or diestrus (Goldman et al., 2007). My initial results from estrous cycle data indicated that there were no estrous cycle differences in ongoing RVM cell activity or heat- and light-evoked activity (Fig 19 and 20, Appendix A). Ongoing response of RVM neurons sets the nociceptive withdrawal threshold, and the stimulus-evoked activity corresponds to time of increased sensitivity (Ramirez et al., 1989, Heinricher et al., 1989). Thus, these results are consistent with findings described above that there are no differences in nociceptive sensitivity between the different phases of the cycle. The studies in this thesis were therefore not powered to draw comparisons between the cycle phases.

4.4 Future directions and clinical implications

4.4.1 Establish light-evoked stimulus-response curves in RVM cells in other pain models

In these studies, hindpaw inflammation served as a model of persistent peripheral pain to determine effects on the photoresponsiveness of RVM cells. CFA was used to induce inflammation because it produces inflammation lasting at least two weeks, and is a standard model of persistent pain (Ren, 1999). Animals develop edema at the injection site and display hypersensitivity to external stimuli and spontaneous pain-related

behaviors (Stein et al., 1988). It would be interesting to determine the effects of CFA-induced orofacial inflammation on RVM cell light-evoked stimulus-response curves. There are important organizational differences between the trigeminal sensory system and the dorsal horn (Bereiter et al., 2000), and, in contrast to hindpaw inflammation, unilateral orofacial inflammation induces contralateral and widespread hypersensitivity that is mediated by RVM (Ambalavanar et al., 2006, Chai et al., 2012, Sugiyo et al., 2005, Shimizu et al., 2009). Additionally, it would be important to investigate the light-evoked stimulus-response curve in a migraine model. These studies would help determine if greater degrees of hypersensitivity or involvement of the trigeminal sensory system result in greater enhancement of the photoresponsiveness of RVM neurons.

4.4.2 Determine the effects of light on nociceptive sensitivity

Previous work demonstrated that light moderately lowers the thermal nociceptive withdrawal threshold (Martenson et al., 2016). However, the only light intensity tested in that study was 18,000 lux, and my data demonstrate that much lower light intensities evoke a response from RVM. Therefore stimulus-response curves should be established for exposure to a range of light intensities on noxious-stimulus evoked withdrawal during light. Additionally, studies are needed in which the RVM is blocked to determine if the RVM directly mediates this light-induced hyperalgesia. Behavioral work in models of persistent inflammation will also be important to establish how light interacts with already established hyperalgesia.

4.4.3 Clinical implications

Photophobia is a common complaint in functional pain disorders such as fibromyalgia and migraine. However it is a poorly understood condition, with limited treatment options. In this thesis I further characterized a mechanism through which the pain-modulation system could contribute to photophobia. Importantly, my findings show that pain-inhibiting OFF-cells are suppressed at a lower light level, and to a greater

extent during persistent peripheral inflammation. These data suggest that, in pain states, decreased descending inhibition of pain during light exposure could contribute to photophobia. My results suggest that light may act through a “top-down” mechanism to modulate the activity of RVM neurons. Since RVM modulates nociceptive transmission at the dorsal horn and trigeminal nucleus, these changes in RVM cell activity may have direct and widespread consequences for somatic sensitivity. Furthermore, in chronic pain disorders, central sensitization, or changes in the central nervous system that contribute to hypersensitivity, are hard to test for and quantify in clinic. My finding that there is dysfunction in central descending control pathways in persistent pain suggests that sensitivity to light could be a potential index of dysfunction in pain-modulation circuitry.

Additionally, chronic pain disorders are more prevalent in females, despite similar nociceptive sensitivity in the two sexes in healthy individuals. Thus, it is likely that there are differences in pain-processing that make females more vulnerable to developing chronic pain. However, my results show that RVM activity, the output of a major pain-modulation system, is similar in the two sexes under basal conditions and in persistent inflammation. Thus, molecular differences in descending pain-modulation from PAG-RVM circuitry likely do not contribute significantly to observed sex differences in clinical conditions.

4.5 Summary of findings

In this thesis, I demonstrated that the physiological output of the descending pain-modulation system has similar properties between male and female animals under basal conditions and in persistent inflammation. Additionally, I established light-evoked stimulus-response curves in RVM that show that RVM cell response to light is graded. Furthermore, in animals with persistent inflammation, the stimulus-response curve of OFF-cells is left-shifted, indicating that light dampens descending pain-inhibition at a

lower threshold and to a greater extent at dim levels of light. Continuing to elucidate how the pain-modulation circuitry responds to different stimuli and under different conditions in both males and females will lead to improved understanding of pain conditions.

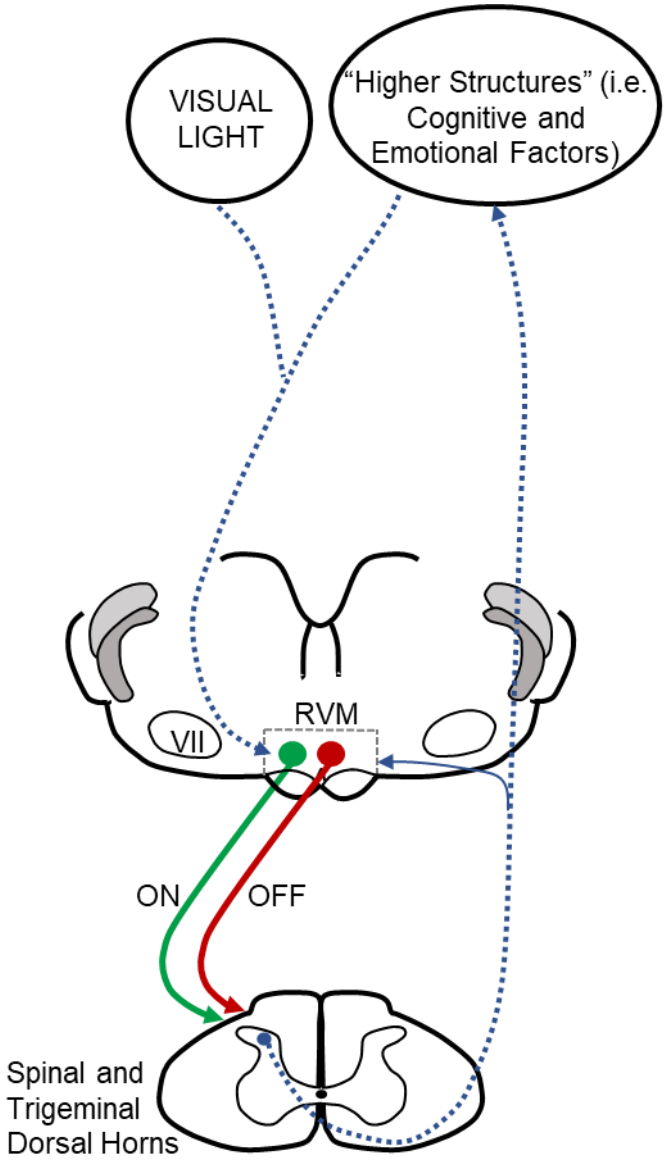

Figure 18 Visual light as a "top-down" input to RVM

Visual light likely exerts a modulatory influence on RVM cell activity, which in turn controls nociceptive transmission through pain facilitatory ON-cells and pain-inhibitory OFF-cells. This mechanism could provide a means for RVM to contribute to photophobia. Dashed lines represent indirect connections.

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APPENDIX A

SUPPLEMENTAL FIGURES

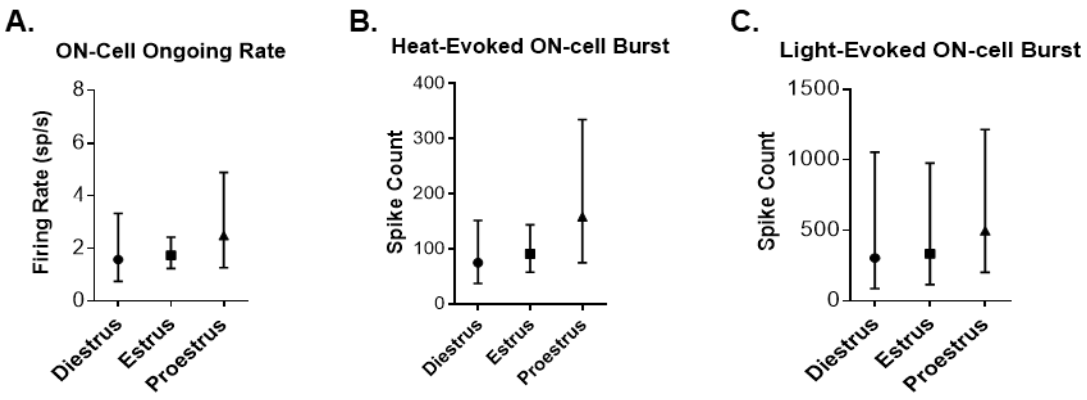

Figure 19 ON-cell activity across different phases of the estrous cycle

There is no significant difference in ON-cell activity between diestrus, estrus, and proestrus based on ongoing activity (A) (Kruskal-Wallis test, H=0.96, p=0.62), heat-related activity (B) (as defined by the total number of action potentials in the heat-evoked “burst”, Kruskal-Wallis test, H=2.43, p=0.30), or light-related activity (C) (as defined by the total number of action potentials in the light-evoked “burst”, Kruskal-Wallis test, H=0.46, p=0.79). Data are displayed as geometric mean +/- 95% CI, n=7-22 cells/group.

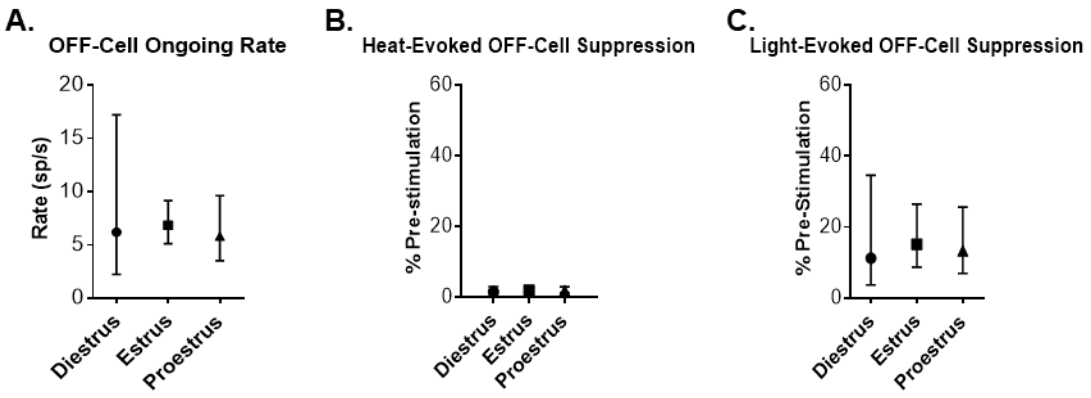

Figure 20 OFF-cell activity across different phases of the estrous cycle

There is no significant difference in OFF-cell activity between diestrus, estrus, and proestrus based on ongoing activity (A) (Kruskal-Wallis test, H=0.31, p=0.86), heat-evoked suppression (B) (as defined by percent firing rate during paw withdrawal relative to the firing rate prior to heat onset, Kruskal-Wallis test, H=1.40, p=0.50), or light-evoked suppression (C) (as defined by percent firing rate during light exposure relative to the firing rate prior to light onset, Kruskal-Wallis test, H=0.53, p=0.77). Data are displayed as geometric mean +/- 95% CI, n=8-21 cells/group.