AN INTERACTION BETWEEN ESTROGEN AND SEROTONIN IN SENSORY NEURONS AS A KEY REGULATOR OF NOCICEPTION

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DEDICATION

To Mamma, Pappa, Reshu, Preet, and Gursimran for constantly motivating me

To Dallas Sangat for their unwavering support

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"We must have perseverance and above all confidence in ourselves. We must believe that we are gifted for something and that this thing must be attained." — Marie Curie

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ABSTRACT

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AN INTERACTION BETWEEN ESTROGEN AND SEROTONIN IN SENSORY NEURONS AS A KEY REGULATOR OF NOCICEPTION

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Orofacial pain conditions, such as migraine, are at least two times more common in women and have a complex etiology. Orofacial pain, relayed by trigeminal sensory neurons, is linked to gonadal hormone (estrogen; progesterone) fluctuations. The neurotransmitter serotonin (5HT) is a proinflammatory and pronociceptive mediator in the periphery and has been implicated in several female prevalent pain disorders. 5HT can directly activate trigeminal sensory neurons or can sensitize the transient receptor potential vanilloid 1 ion channel (TRPV1), a cation channel activated by capsaicin and heat. TRPV1 activation results in calcium influx and calcitonin gene-related peptide (CGRP) release, leading to peripheral sensitization that heightens pain sensitivity. Previous studies in male rats have shown that 5HT receptors colocalize with and sensitize TRPV1. Furthermore, serotonergic potentiation of CGRP release occurs from the dental pulp of women during the luteal phase of the menstrual cycle (when gonadal hormones are fluctuating). It is unknown whether estradiol (E2; primary estrogen) enhances or attenuates this serotonergic pain mechanism. As >90% of pain research has been conducted in males, and a small subset examines the trigeminal system, gonadal hormone modulation of female trigeminal sensory neurons is grossly understudied and warrants investigation. Our overarching hypothesis is that hormone status alters serotonergic neuromodulation of the TRPV1-expressing subpopulation

of trigeminal sensory neurons. Thus, we examined whether (1) naturally fluctuating gonadal hormones can alter 5HT-evoked pain behaviors, (2) varying E2 concentration and exposure time can alter trigeminal pain signaling and transcriptome, and (3) E2 and estrogen receptors (ERs) can modulate the pronociceptive effects of 5HT on TRPV1 function. We report that exogenous 5HT evokes significant pain behaviors in females during proestrus and estrus. 5HT2A receptor antagonism or a steady-state diestrus level E2 attenuates 5HT-evoked pain behaviors. We also report that ERα, ERβ, and G protein coupled ER (GPER) localize to sensory neurons expressing 5HT2A and TRPV1 mRNA. Lastly, E2 modulates specific trigeminal pain genes and enhances ERα-dependent serotonergic potentiation of CGRP release. Together, the data presented in this dissertation provides behavioral, neuroanatomical, and cellular evidence of a mechanism present in female trigeminal sensory neurons that may exacerbate trigeminal pain disorders in women.

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CHAPTER I

INTRODUCTION

Pain, Nociception, and Pain Disorders

According to the International Association for the Study of Pain, pain is defined as an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage (Raja et al., 2020). Pain processing involves the initial detection of incoming pain signals by the peripheral sensory nerve endings, termed nociceptors (Deuis et al., 2017) . Nociceptors are the unmyelinated nerve endings of peripheral afferent axons (Aδ or C fibers that conduct signals to the spinal cord or brainstem) and respond to a variety of incoming noxious mechanical (touch, pressure), thermal (extreme hot or cold), and/or chemical (inflammatory mediators, protons) stimuli by generating action potentials (Averitt et al., 2019; Loyd et al., 2013). The cell bodies of nociceptors are located in either the dorsal root ganglia (DRG) or the trigeminal ganglia (TG). The peripheral branches of the DRG innervate the trunk, viscera, and extremities, and synapse in the spinal dorsal horn (see Figure 1.1A). Whereas the peripheral branches of the TG innervate the cranial and orofacial region and terminate in the medullary dorsal horn (see Figure 1.1B). From the spinal and medullary dorsal horn, the pain signals are then relayed via the thalamus to the cortex (see Figure 1.1C), creating a perception of pain and integrating the peripheral pain signals to the central nervous system (DeLeo, 2006; Julius & Basbaum, 2001; Sessle, 2011). While pain is a subjective experience and is influenced by hormonal, pathological, immunological, emotional, and sociocultural factors, nociception is purely defined as the detection of potentially noxious stimuli followed by an autonomic or a

behavioral response (Mischkowski et al., 2018; Nasser & Afify, 2019; Sneddon, 2018). Pain can be classified as acute, chronic, somatic, visceral, or neuropathic depending on the duration and severity of pain (Nasser & Afify, 2019). National Academy of Sciences reports that more than 100 million Americans experience chronic pain and the medical treatment for such conditions costs approximately $635 billion per year (Simon, 2012). Decades of research has indicated that chronic pain conditions such as irritable bowel syndrome, chronic fatigue syndrome, interstitial cystitis, low back pain, neck pain, knee pain, osteoarthritis, fibromyalgia, temporomandibular join disorder, migraine, and headaches are sexually dimorphic and have a marked female prominence (Cairns & Gazerani, 2009; Fillingim et al., 2009; Greenspan, Craft, LeResche, Arendt-Nielsen, Berkley, Fillingim, Gold, Holdcroft, Lautenbacher, Mayer, Mogil, Murphy, Traub, et al., 2007; Leresche, 2011; Mogil, 2012).

Trigeminal Pain Disorders and Sex Differences

Sexually dimorphic pain disorders such as migraine, headache, fibromyalgia, and temporomandibular joint disorder (TMD), involve activation of the sensory neurons of the trigeminal ganglia (Berkley, 1997; Dao & LeResche, 2000; LeResche, 1997). They are classified as orofacial pain conditions and affect both, males, and females. Pre-puberty, the incidence of orofacial pain conditions is almost comparable in both sexes. But, post-puberty, the pain is more frequent, severe, and longer lasting in women (Cairns & Gazerani, 2009; Delaruelle et al., 2018). Based on population studies, TMD is at least 2–5 times more common in women, migraine is at least 2 times more common in women, and tension-type headache is 1.5 times more common in women (LeResche et al., 2003a; Lyngberg et al., 2005; Medicine, 2011; Moloney et al., 2016). In addition, even though fibromyalgia affects only 2–3% of the population, 80% of the cases are reported in women (Staud, 2006). Despite their prevalence in women, the exact mechanism(s) underlying orofacial pain conditions remains elusive. Thus, it is vital to understand the underlying

cause of this sexual dimorphism for effective treatment of chronic pain conditions. Since inflammatory mediators and gonadal hormones have been implicated in trigeminal pain conditions, the current literature investigating these interactions will be explored in the next sections.

Inflammation and Peripheral Sensitization

A common hallmark of peripheral pain conditions is the recruitment of immune cells at the site of nociceptor activation. Inflammation recruits immune cells namely, T cells, mast cells, macrophages, and blood platelets to the injury site, which triggers release of various proinflammatory mediators like bradykinin, serotonin (5-hydroxytryptamine; 5HT), protons, nerve growth factor (NGF), and ATP (Ji et al., 2014). These inflammatory mediators can then bind to their specific receptors on the trigeminal nociceptors, and further enhance the activity of nociceptors. Nociceptors express numerous types of receptors that modulate rapid signaling in the primary afferent neurons. Some examples of these receptors include the G-protein coupled receptors, ion channels, hormone receptors, and tyrosine kinase receptors. A major class of ion channels that transduce pain in the nociceptors are the transient receptor potential (TRP) superfamily (Stucky et al., 2009). TRP ion channels are multimodal and are activated by a wide range of noxious temperatures (hot and cold) and chemical stimuli (Stucky et al., 2009). A major thermosensor TRP channel that is expressed by a subset of TG and DRG neurons is the Transient Receptor Potential Vanilloid 1 (TRPV1) ion channel (Holzer, 1988). TRPV1 is activated by noxious heat (> 42°C), capsaicin (CAP; the active compound in chili peppers), protons (released during inflammation), and endogenous ligands like linoleic acid and oxytocin (Caterina et al., 1997; Nersesyan et al., 2017; Patwardhan et al., 2010; Tominaga et al., 1998). Structurally, the ion channel superfamily has 6 transmembrane domains with cytosolic N and C termini and a pore forming hydrophobic domain (Caterina et al., 1997; Stucky et al., 2009). TRPV1 activation

results in transient calcium (Ca^{2+}) influx within the cell and post-synaptic release of proinflammatory mediators, including calcitonin gene related peptide (CGRP) and substance P, further enhancing the pain signals by causing peripheral sensitization (Immke & Gavva, 2006; Sluka et al., 1992; Willis, 2009). Peripheral sensitization by inflammatory mediators results in reduction of the sensory threshold required for activation of TRPV1. TRPV1 can also be sensitized due to phosphorylation by cellular kinases such as Protein Kinase A (PKA), Protein Kinase C (PKC), Ca^{2+} dependent calmodulin kinase II (CaMK II), and cyclin dependent kinase 5 (Cdk5; Caterina et al., 1997; Dhaka et al., 2006; Premkumar & Ahern, 2000; Sluka et al., 1992; Stucky et al., 2009; Sugiuar et al., 2004; Tominaga & Caterina, 2004). Phospholipase C is also known to sensitize TRPV1 by degrading membrane bound phosphoinositol-4, 5-bisphosphate (PIP_2) to form secondary messenger diacylglycerol (DAG) and inositol-1, 4, 5-triphosphate (IP_3), thereby relieving PIP_2 inhibition of TRPV1 (Chuang et al., 2001; Stucky et al., 2009).

Serotonin and Trigeminal Pain

The vast majority of trigeminal pain conditions share a role of 5HT in the underlying pain mechanism, including migraine (Chen & Ashcroft, 2008; Ferrari et al., 2001), TMD (K Okamoto et al., 2005), and fibromyalgia (Häuser et al., 2009). The degree of importance of 5HT in trigeminal pain is illustrated by the observation that 99% of 5HT is found outside the central nervous system. 5HT is a well-known mood and appetite regulatory neurotransmitter in the central nervous system. However, it is a proinflammatory and pronociceptive mediator in the periphery (Asghari et al., 2011). In the periphery, 5HT is synthesized by intestinal enterochromaffin cells, neuroendothelial cells, (Herr et al., 2017; Ni et al., 2008), lymphocytes, macrophages, mast cells, platelets, and monocytes (Mössner & Lesch, 1998). Enterochromaffin cells are the predominant sources of 5HT and synthesize up to 95% of the total 5HT in the body (Ni et al., 2008). The peripheral 5HT is majorly sequestered and stored in platelets (up to 65 mM)

and mast cells and released upon activation (Holmsen & Weiss, 1979). Interestingly, exogenous injection of 5HT into the female human masseter muscle induces local pain and allodynia (Ernberg et al., 2006) and injection of 5HT in male rat paw also elicits inflammation and hyperalgesia (Taiwo & Levine, 1992). Furthermore, in cultured rat TG neurons, application of 5HT upregulates the expression of TRPV1 ion channels, thus strengthening the Ca^{2+} responses in the neurons (Simonetti et al., 2006). In support, 5HT significantly enhances membrane excitability and CAP-evoked currents in cultured mouse DRG neurons (Sugiuar et al., 2004). Lastly, pretreatment of rat TG neurons with 5HT significantly enhanced CAP-evoked Ca+2 influx and CGRP release, indicating a modulatory role of 5HT on TRPV1 function (Loyd et al., 2011).

Estrogen and Trigeminal Pain

Since orofacial pain conditions are more prominent in women, another possible explanation is the effect that fluctuating gonadal hormones (estrogen and progesterone) may exert on pain processing (Kuba & Quinones-Jenab, 2005). The fluctuations in the levels of gonadal hormones during puberty, the menstrual cycle, and pregnancy tend to exacerbate trigeminal pain conditions (P. R. Kramer & L. L. Bellinger, 2009; LeResche et al., 2003a). Progesterone has a well-documented anti-inflammatory role whereas estrogen's (E2) effect on pain processing is controversial (Averitt et al., 2019; Charlet et al., 2008; Craft, 2007). While studies report that high levels of serum E2 are linked to TMD, low levels of E2 are reported to precipitate a migraine attack (Craft, 2007). Furthermore, clinical studies report half of women experience menstrual-associated migraine, pain associated with fibromyalgia is highest during the luteal phase of the menstrual cycle when E2 levels are high, and the incidence of TMD pain increases in post-menopausal women undergoing E2 replacement therapy (Cairns & Gazerani, 2009; Craft, 2007). The literature predominately reports that E2 is pronociceptive. In contrast, Pavlović et al.

(2016) reported that migraine patients can be characterized by a rapid drop in E2 as compared to controls, suggesting an important role of the timing and concentration of E2. The effects of E2 on trigeminal pain have been studies in animal models as well. Comparable to human menstrual cycle (see Figure 1.2A), rats have a four-day estrous cycle (see Figure 1.2B), with rapid gonadal hormone fluctuations occurring during the proestrus and estrus phases of the cycle (comparable to luteal phase). In female rats, increased visceral hypersensitivity (increased response to a noxious stimulus) is seen during the proestrus and estrus phases of the estrous cycle (Moloney et al., 2016). Additionally, pain behaviors and visceral sensitivity were higher in female rats during proestrus and estrus (Moloney et al., 2016). In ovariectomized rats, high E2 significantly enhanced nocifensive behaviors and increased the expression of TRPV1 in TG (Yamagata et al., 2016). Taken together, these data indicate that E2 can modulate the trigeminal pain in a concentration and timing dependent manner and warrants further investigation into the specific receptors that may play a role in this modulation.

Estrogen Modulates Peripheral Serotonin

Reports from a variety of fields indicate that E2 modulates physiology via 5HT; including vasodilation, clotting, recruitment of immune cells, gastrointestinal motility, lordosis, and initiation of uterine contractions (Rybaczyk et al., 2005; Uphouse et al., 2011). Specifically, an increase in E2 leads to an increase in plasma 5HT (Blum et al., 1996) and tryptophan hydroxylase (rate-limiting enzyme for 5HT synthesis; Bethea et al., 2000), while both reducing and antagonizing the serotonin reuptake transporter (SERT), in humans and macaques (Ofir et al., 2003; Pecins-Thompson et al., 1998). Also, Benmansour et al. (2016) has shown that externally administered E2 can maintain high levels of 5HT in the periphery in ovariectomized (OVX) rat. In the central nervous system, E2 is shown to regulate the transcription of 5HT synthesizing enzyme, tryptophan hydrozylase-2 via the estrogen response elements in the promoter sequence

of the gene (Hiroi & Handa, 2013). In support, E2 increases trigeminal nociceptor excitability and sensitization by inflammatory mediators in cultured TG neurons (Rowan et al., 2014). As 5HT is proinflammatory and pronociceptive in the periphery, the effects of fluctuating E2 on the serotonergic system have clear implications in the prevalence of trigeminal disorders in women.

Estrogen, Serotonin, and TRPV1 Sensitization

Since E2 can modulate the trigeminal serotonergic system, it is essential to understand the role of specific estrogen receptors (ER) and 5HT receptors in trigeminal pain processing. 5HT can act via the seven currently known classes of 5HT receptors (5HTR; $5HT_1$-$5HT_7$) to alter electrical conductivity or to activate numerous downstream signaling cascades. Of the seven classes of 5HT receptors, only $5HT_3$ receptors are ionotropic whereas other families are G-protein coupled receptors (GPCR). $5HT_1$ and $5HT_5$ receptors activate the inhibitory $G_{i/o}$ subunit and the rest activate excitatory G_s or $G_{q/11}$ subunits (Hoyer et al., 2002). Some of the 5HT GPCRs can trigger pain via activating signaling cascades known to sensitize TRPV1. Studies have shown that inhibitory $5HT_{1B}$ and $5HT_{1D}$ receptors and excitatory $5HT_{2A}$ and $5HT_3$ receptors co-localize on trigeminal sensory neurons that express TRPV1 in male rats. Activation of the excitatory receptors enhances capsaicin-evoked Ca^{+2} influx and CGRP release in cultured TG neurons. This enhancement is attenuated in the presence of $5HT_{1B}$ and $5HT_{1D}$ receptor agonists or $5HT_{2A}$ and $5HT_3$ receptor antagonists (Loyd et al., 2012; Loyd et al., 2011). Potentiation of TRPV1 by 5HT in the peripheral nervous system has also been studied in irritable bowel syndrome (IBS) that causes visceral hypersensitivity. Sugiuar et al. (2004) showed that in the presence of 5HT, capsaicin-, heat-, and proton- evoked currents are significantly higher in mouse DRG neurons from male rats. These currents are attenuated in the presence of $5HT_2$ and $5HT_4$ receptor antagonists and in the presence of PKA and A kinase anchoring protein (AKAP) inhibitors. Evidently, these effects have been studied in male rats despite the prevalence or trigeminal pain

conditions and IBS in females. Gonadal hormones can also exert their effect via nuclear or membrane-bound ERs to modulate 5HTs role on trigeminal pain processing. While the nuclear ERs, ERα and ERβ classically act via binding to promoter sequences of genes that contain E2 response element (EREs), G-protein coupled membrane ER (GPER) act via activating downstream kinases in the cell (Chen et al., 2021). Further, E2 can directly modulate TRP channels. A study by Irnaten et al. (2008) showed that E2 causes a rapid Ca^{+2} influx into the epithelial cells via TRPV6 channel, and Yamagata et al. (2016) showed that in female TGs, E2 upregulates the expression of TRPV1 mRNA (Bereiter et al., 2005; Craft, 2007; Flake et al., 2006; Ji et al., 2018; Rowan et al., 2014). Thus, gonadal hormones can act through receptors present on the peripheral nerve endings to activate downstream signaling cascades that may play a role in sensitizing the nociceptors. In a study involving human dental pulp, CAP-evoked CGRP release was assessed in tooth pulp cultures from men and women. When preteated with 5HT, CAP-evoked CGRP release was significantly higher in dental pulp from women, whereas CGRP release was comparable to saline control in dental pulp from men. When stratified for the menstrual cycle stage, it was observed that greatest amount of CGRP release occurred from tooth pulp obtained during the luteal phase of the cycle (Loyd et al., 2012a). These studies indicate that E2 can modulate 5HTs pronociceptive effects in the trigeminal sensory neurons.

Rattus norvergicus- A Model to Study Trigeminal Pain Disorders

Rat serves as a great model system to study trigeminal pain disorders since a lot of orofacial pain behaviors have been characterized in the rat (Krzyzanowska & Avendano, 2012) and it has a similar distribution of the trigeminal nerves with the V1 innervating the ophthalmic region, V2 innervating the cheek and whisker pad, whereas V3 sending sensory neurons in the lower face and jaw region (see Figure 1.3). Furthermore, numerous migraine analgesic studies have been well characterized in rats, making it a great model system to understand peripheral pain

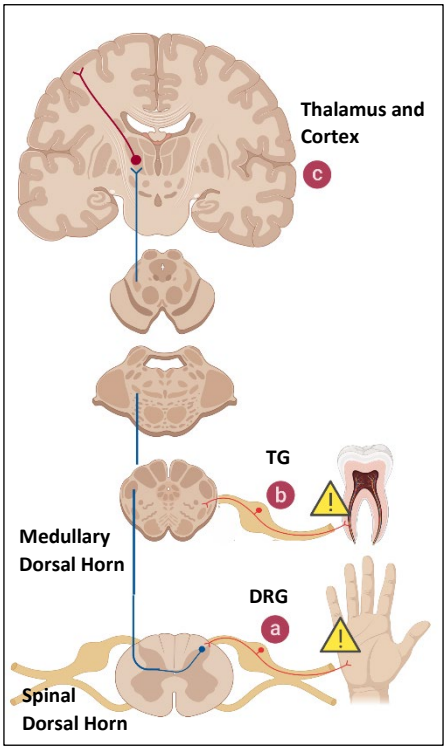
processing and analgesia (Pelissier et al., 2002). Lastly, orofacial nocifensive behaviors used in this dissertation have been well-characterized in response to various pruritogens and algogens (Spradley et al., 2012).

Significance

Overall, these data indicate that E2 and 5HT may interact to modulate pain processing in females. But, the exact receptors and signaling cascades involved in the modulation are not known. Also, 5HT-evoked pain behaviors have not been studied over different controlled E2 concentration. Our study aims to elucidate the molecular mechanisms underlying 5HT mediated sensitization of TRPV1 and the possible influence of E2 fluctuations in this sensitization. Thus, our overarching hypothesis is that hormone status alters serotonergic neuromodulation of the TRPV1-expressing subpopulation of trigeminal sensory neurons. Chapter II of this dissertation employs *in vivo* hindpaw pain behavior testing to assess the role of 5HT on peripheral pain processing. Chapter III utilizes *in vivo* orofacial pain behavior testing combined with *ex vivo* cell culture experiments to address the role of 5HT on trigeminal pain during natural hormonal fluctuations. We also report the importance of excitatory $5HT_{2A}$ receptor in peripheral pain processing in both the chapters. Chapter IV focuses on the role of an additional excitatory 5HT receptor, $5HT_{3A}$ in trigeminal pain processing. Chapter V aims on manipulating and controlling estrogen levels to evaluate how the timing and concentration of E2 exposure alters 5HT-evoked pain and plasticity in the underlying anatomical substrate. We also report on changes in the transcriptome of the trigeminal sensory neurons during E2 exposure. Furthermore, Chapter V also analyzes the functional output of the receptor interaction and sensitization in pain processing. This dissertation then concludes with a thorough discussion of our results (Chapter VI), how our work relates to the current literature in light of a model that explains the particular mechanism that we provide evidence for to account for the prevalence of trigeminal pain disorders in women.

Figure 1.1

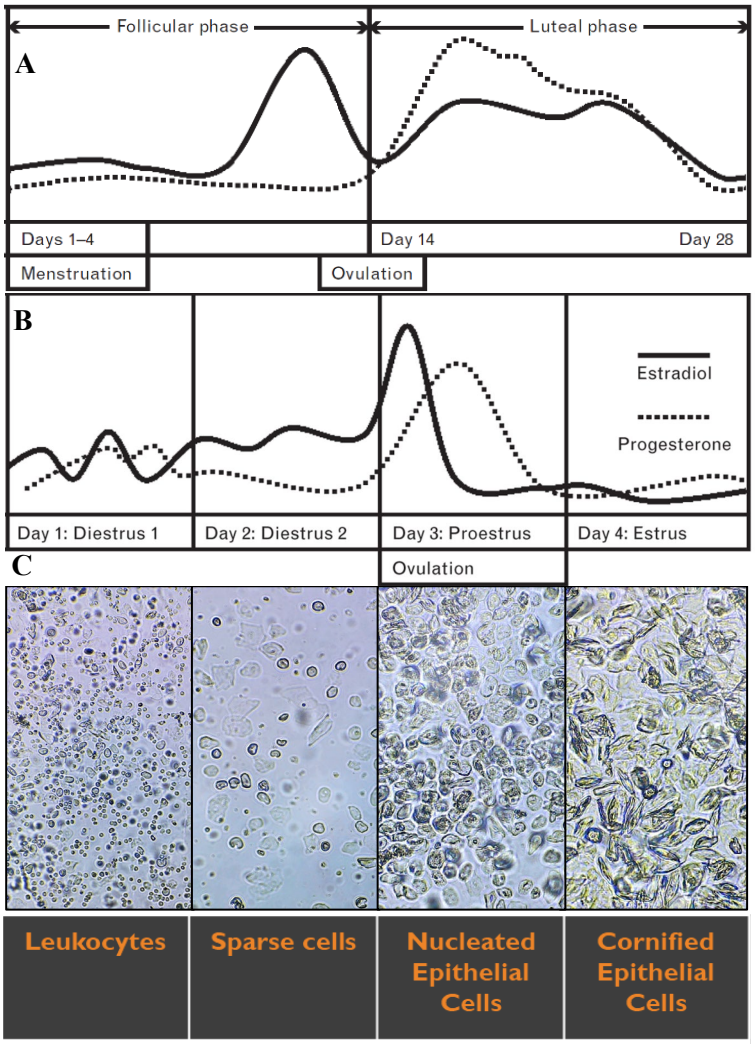
Peripheral and Central Pain Processing Pathways



Note. Pain Processing Pathways via the (A) Dorsal Root Ganglia and (B) Trigeminal Ganglia, Connecting to the (C) Higher Brain Regions (Adapted from Averitt et al., 2019).

Figure 1.2

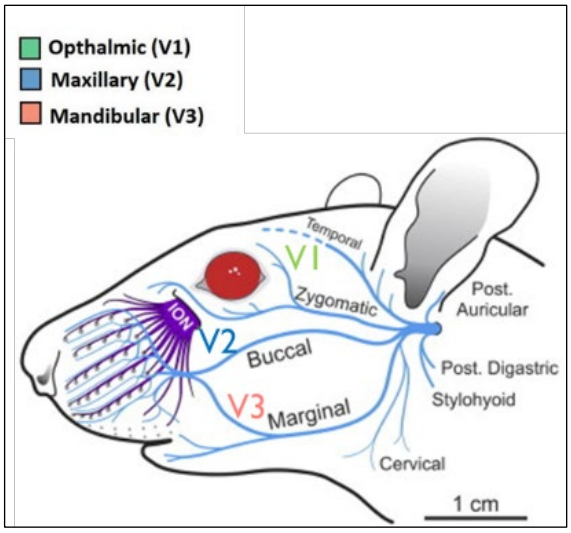
Comparison of Human Menstrual Cycle and Rat Estrous Cycle



Note. Gonadal Hormone Fluctuations during the (A) Human Menstrual Cycle, (B) Rat Estrous Cycle, and (C) Distinct Cell Populations in the Rat Estrous Cycle (Adapted from Chai et al., 2014)

Figure 1.3

Rat Trigeminal Ganglia Anatomy



Note. Trigeminal Ganglia Branches (V1, V2, V3) Innervating the Rat Orofacial Region (Adapted from Heaton et al., 2014)

CHAPTER II

SEX DIFFERENCES AND ESTROUS CYCLE EFFECTS OF PERIPHERAL SEROTONIN-EVOKED RODENT PAIN BEHAVIORS

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Abbreviations

5HT: serotonin

$5HT_{2A}$ receptor: serotonin 2A receptor subtype

$5HT_3$ receptor: serotonin 3 receptor subtype

ANOVA: analysis of variance

CGRP: calcitonin gene-related peptide

DMSO: dimethyl sulfoxide

ELISA: enzyme-linked immunosorbent assay

GPCR: G protein-coupled receptor

IBS: irritable bowel syndrome

ipl: intraplantar

M100907: serotonin 2A receptor antagonist

PWL: paw withdrawal latency

TMD: temporomandibular joint disorder

TRPV1: transient receptor potential vanilloid 1 ion channel

Highlights

Peripheral serotonin evokes greater and longer-lasting thermal hyperalgesia in female rats in proestrus and estrus compared to male rats, ovariectomized female rats, or females in diestrus.

Serotonin evokes mechanical allodynia in female rats in proestrus and estrus, but not in males, ovariectomized females, or females in diestrus.

No sex differences or estrous cycle effects were observed in 5HT-evoked hindpaw edema or 5HT content in the interstitial fluid of CFA-inflamed hindpaws.

Local injection of the $5HT_{2A}$ receptor antagonist M100907 blocked 5HT-evoked pain behaviors in cycling female and male rats.

Abstract

Many persistent pain conditions occur predominantly in women making pain a major women's health issue. One theory for the prevalence in females is hormone modulation of pain mechanisms. The peripheral release of the neurotransmitter serotonin (5HT) has been implicated in various sexually dimorphic pain conditions; yet no studies have examined the effect of ovarian hormones on peripheral 5HT-evoked pain behaviors. We hypothesized that peripheral 5HT evokes greater pain behaviors in female rodents during estrus and/or proestrus, stages of the estrous cycle where ovarian hormones are greatly fluctuating. Female Sprague-Dawley rats (250-350 g) from each stage of the estrous cycle, ovariectomized females, and intact males received an intraplantar hindpaw injection of 5HT (2 μg / 100 μL) or saline (n = 6 per group) and thermal hyperalgesia, mechanical allodynia, or edema was measured at 0, 10, 20 and 30 minutes post-injection. A separate group of rats received an ipsilateral injection of the selective $5HT_{2A}$ antagonist, M100907, 15 minutes prior to 5HT injection. We report that females in proestrus and estrus exhibited significantly greater and/or longer lasting pain behaviors compared to males, females in diestrus, and ovariectomized females. There were no significant sex differences or estrous cycle effects on 5HT-evoked edema or 5HT content in inflamed hindpaws. Local pretreatment with the $5HT_{2A}$ receptor antagonist blocked 5HT-evoked thermal hyperalgesia and edema. These data provide evidence of a modulatory role of hormones on peripheral 5HT-evoked pain occurring via the $5HT_{2A}$ receptor.

Keywords: *pain; serotonin; estrous cycle; $5HT_{2A}$ receptor; sex differences; edema*

Introduction

Sex differences are seen in pain conditions such as fibromyalgia, irritable bowel syndrome (IBS), migraine, and temporomandibular joint disorder (TMD) pain [1, 2]. These conditions are more prevalent in women as compared to men with women reporting a longer duration and higher intensity of pain [3] . Based on population studies, the female: male ratio is 2:1 for migraine and 3:1 for IBS. Additionally, 80% of the treated TMD cases are females. Medical treatment of such chronic pain conditions is estimated to be approximately $635 billion per year [4]. Thus, understanding the underlying cause of this sexual dimorphism is vital for its effective treatment.

Consistent with the hypothesis that fluctuating gonadal hormones play an important role in these differences, half of women report menstrual-associated migraine [5], pain associated with fibromyalgia is highest during the luteal phase of the menstrual cycle when estrogen levels are high [6], and the incidence of TMD pain increases in postmenopausal women undergoing estrogen replacement therapy [7]. In female rats, increased visceral hypersensitivity is seen during the proestrous and estrous stages of the estrous cycle [3]. Also, the major active estrogen, 17-β estradiol, can regulate the release of the proinflammatory mediator calcitonin gene-related peptide (CGRP) in cultured cells *in vitro* [8]. Thus, gonadal hormones can act through receptors present on the peripheral nerve endings to activate downstream signaling cascades that may play a role in sensitizing the nociceptors.

Another important factor that may exacerbate the pain condition is the presence of inflammation. Inflammation involves recruitment of immune cells, mast cells, and blood platelets to the injury site, which then trigger release of a plethora of various inflammatory mediators including CGRP, serotonin (5-hydroxytryptamine; 5HT), bradykinin and prostaglandins [9, 10]. 5HT is a well-known mood and appetite regulatory neurotransmitter in the central nervous

system. However, it is a proinflammatory and pronociceptive mediator in the periphery. Exogenous injection of 5HT into the female human masseter muscle induces local pain and allodynia [11] and injection of 5HT in male rat paw also elicits inflammation and hyperalgesia [12]. 5HT is also shown to contribute to visceral hypersensitivity of primary afferent neurons in IBS [13].

5HT can act via the seven currently known classes of 5HT receptors, $5HT_{1\text{-}7,}$ to alter electrical conductivity or to activate numerous downstream signaling cascades. Most 5HT receptors, with the exception of $5HT_3$ which is an ionotropic receptor [14], are metabotropic and act via G protein-coupled receptors (GPCRs). Some of the 5HT GPCRs can trigger pain via activating signaling cascades known to sensitize the transient receptor potential vanilloid 1 (TRPV1) ion channel, a thermosensor expressed by a major subset of sensory neurons that is activated by noxious heat (>42°C), capsaicin, and protons. Activation of TRPV1 channels causes a transient calcium influx and subsequent release of pronociceptive mediators, including CGRP [15, 16], to trigger and contribute to peripheral sensitization. Of the 5HT receptors localized on nociceptors, the $5HT_{2A}$ appears to be the most involved in pain and sensitization of TRPV1 [13, 17-21]. Application of topical capsaicin in humans is associated with a greater increase in pain intensity in females as compared to males [22]. In support, higher intensity of pain is reported in females in the menstrual phase compared to the luteal phase after an intradermal capsaicin injection [23].

Serotonin increases capsaicin-evoked CGRP release from rat sensory neurons via the peripheral $5HT_{2A}$ and $5HT_3$ receptors (Loyd et al., 2011; Loyd et al, 2012). These receptors are co-expressed with TRPV1 on sensory neurons providing an anatomical substrate for enhancing pain signaling in these cells [21]. This potentiation of TRPV1 by 5HT has also been demonstrated in human nociceptors in dental pulp extracted specifically during the luteal phase of the menstrual

cycle [19], indicating a potential modulatory role of hormones on this pain mechanism. Reports from a variety of fields indicate that estrogen is modulating physiology via 5HT; including vasodilation, clotting, recruitment of immune cells, gastro-intestinal motility, lordosis, and initiation of uterine contractions [see 24, 25]. The degree of importance of 5HT in these functions is illustrated by the observation that 99% of 5HT is found outside the central nervous system. Specifically, an increase in estrogen leads to an increase in plasma 5HT [26] and tryptophan hydroxylase (rate-limiting enzyme for 5HT synthesis) [27], while both reducing and antagonizing the serotonin reuptake transporter [28, 29], in humans and macaques. As 5HT is proinflammatory and pronociceptive in the periphery, the effects of fluctuating estrogen on the serotonergic system have clear implications in the prevalence of pain disorders involving 5HT in women.

Despite the prevalence of pain disorders in women, the underlying mechanisms linking fluctuating ovarian hormone levels with pain remain elusive. The vast majority of pain conditions that are more prevalent in women share a role of 5HT in the underlying pain mechanism, including migraine [30, 31], TMD [17], IBS [32], and fibromyalgia [33, 34]. To date, no study has examined the relationship between the effects of peripheral 5HT and stage of menstrual/estrous cycle on pain behaviors in females. Here we hypothesized that peripheral 5HT evokes greater pain behaviors during periods of the rat estrous cycle when gonadal hormones are in flux.

Experimental procedures

Subjects

A total of 49 adult male and 173 adult female Sprague-Dawley rats (250–350 g; Charles River Laboratories, Wilmington, MA) were used in these experiments. Rats were separated by sex and pair-housed in a 12:12 hour light: dark cycle with *ad libitum* access to food and water. All studies were approved by the Texas Woman's University Institutional Animal Care and Use

Committee and conform to federal guidelines and guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain. This study was conducted in strict compliance with the Animal Welfare Act, implementing Animal Welfare Regulations, and the principles of the Guide for the Care and Use of Laboratory Animals.

Vaginal Cytology

Vaginal lavages were performed between 0900_{AM} to 1100_{AM} at 24-hour intervals beginning two weeks (at least two consecutive cycles) before testing to confirm that all female rats were cycling normally and to keep daily records on the stages of their cycle in respect to experimental testing. Proestrus was identified as a predominance of nucleated epithelial cells and estrus was identified as a predominance of cornified epithelial cells. Diestrus 1 (or metestrus) was differentiated from diestrus 2 (or diestrus) by the presence of leukocytes [35-37]. When no significant differences were noted in behavior of diestrus 1 and diestrus 2 animals, these data were pooled and reported as such.

Ovariectomy

Female rats (n=25) were deeply gas anesthetized (3% induction; 2.5% maintenance) by inhalation of Isothesia (isoflurane, USP, Henry Schein Animal Health, Dublin, OH) and a single incision was made across the abdomen. The abdominal muscle was opened and the ovary bundles were ligated with 4-O silk sutures, excised, and removed, as previously described [38]. The fascia was closed with 5-O silk suture and the skin was closed with Vicryl sutures to prevent wicking. Rats were allowed two weeks for recovery and ovarian hormone dissipation.

Drugs

Serotonin hydrochloride (Sigma-Aldrich, St. Louis, MO) was dissolved in double-distilled water and diluted in 0.9% sterile saline immediately prior to each use. The $5HT_{2A}$ antagonist, M100907, was dissolved in 20% dimethyl sulfoxide (DMSO) in 0.9% sterile saline

stored as a 2 mM stock solution at 4°C then serial diluted the day of use to a final working solution of 6% DMSO in 0.9% sterile saline. Immediately prior to use, complete Freund's adjuvant (CFA; Sigma-Aldrich) was dissolved 1:1 in 0.9% sterile saline.

Thermal Hyperalgesia

Basal sensitivity to a noxious thermal stimulus was assessed using the Plantar Test (Ugo Basile; Collegeville, PA)**.** For this test, rats were placed in a clear Plexiglas box resting on an elevated glass plate. Subsequent to acclimation, a beam of light was positioned under the right hindpaw and the average time for the rat to withdraw the paw from the thermal stimulus over three trials, performed at an inter-trial interval of 2–3 min, was recorded in seconds and averaged as the paw withdrawal latency (PWL). The light beam's intensity was set to produce basal PWLs of approximately 15 seconds. A maximal PWL of 20 seconds was used to prevent tissue damage. Twenty-four hours following acclimation, rats received an intraplantar (ipl) injection of either 5HT (2 μg / 100 μL; 30 gauge) or vehicle control (0.9% sterile saline; n = 6–8 per sex, stage of estrous cycle, and experimental condition) into the right hindpaw and thermal hyperalgesia was assessed at 0, 10, 20 and 30 minutes post-injection. A separate group of rats received an injection of the selective $5HT_{2A}$ antagonist, M100907 (0.15 μg / 100 μL; ipl), or vehicle control (100 μL 6% DMSO and 0.9% sterile saline; per sex, stage of estrous cycle, and experimental condition) 15 minutes prior to 5HT injection (2 μg / 100 μL) and thermal hyperalgesia was assessed at 10 minutes post-5HT injection. A control group of female rats (n = 6) received a contralateral (left) hindpaw injection of M100907 (0.15 μg / 100 μL; ipl) 15 minutes prior to 5HT injection (2 μg / 100 μL) and thermal hyperalgesia in the right hindpaw was assessed at 10 minutes post-5HT right hindpaw injection. The time course and concentration of 5HT-evoked thermal hyperalgesia was chosen based on previous studies [12, 19]. The time course and concentration of M100907 was chosen for selectivity based on previous studies reporting binding affinity (Ki = 1.92 nM) of

M100907 for the $5HT_{2A}$ receptor (https://kidbdev.med.unc.edu/databases/pdsp.php). Thermal hyperalgesia testing was conducted by an observer blinded to the experimental condition.

Mechanical Allodynia

A Dynamic Plantar Aesthesiometer (Ugo Basile; Collegeville, PA) was used to elicit a paw withdrawal from a non-noxious stimulus to test for mechanical allodynia as previously described [39]. For this test, rats were placed in a Plexiglas box on an elevated grid platform and a blunt mechanical stimulus was applied to the plantar surface of the left hindpaw. The force (in grams) of the mechanical stimulus was increased with a ramp of 3 grams per second over 10 seconds, with a cutoff of 30 seconds to avoid mechanical lifting of the paw by the device. To reduce the total numbers of animals used in behavior testing, the same rats tested for thermal hyperalgesia, with the exception of the six contralateral injection control rats, were used for mechanical allodynia testing. Mechanical allodynia testing occurred 24 hours following thermal hyperalgesia testing to avoid behavioral sensitization. 48 hours following acclimation, rats received one injection of either 5HT (2 μg / 100 μL; 30 gauge; ipl) or vehicle control (0.9% sterile saline; n = 6–8 per sex, stage of estrous cycle, and experimental condition) into the left hindpaw and mechanical allodynia was assessed at 0, 10, 20 and 30 minutes post-injection. Experimental (5HT injection) and control (saline injection) groups were counterbalanced to avoid an effect of previous 5HT-evoked pain experience on mechanical allodynia. Mechanical allodynia testing was conducted by an observer blind to the experimental condition.

Plethysmometry

In a group of rats separate from behavior testing, hindpaw edema was quantified by electronically measuring changes in paw size by volume displacement with a plethysmometer (paw volume meter; Ugo Basile, Collegeville, PA). Measures of paw volume were observed prior to injections, then every 10 minutes for 60 minutes following injection of 5HT (2 μg / 100 μL; 30

gauge; ipl) or vehicle control (0.9% sterile saline; n = 6 per sex, stage of estrous cycle, and experimental condition) into the right or left hindpaw. Experimental (5HT) and control (saline) injections were counterbalanced to avoid a potential confound of a single rat getting both a left and right hindpaw injection of 5HT. A separate group of rats received a left or right hindpaw injection of the selective $5HT_{2A}$ antagonist M100907 (0.15 μg / 100 μL; ipl) or vehicle control (100 μL 6% DMSO and 0.9% sterile saline; n = 6–8 per sex and experimental condition; counterbalanced) 15 minutes prior to 5HT injection (2 μg / 100 μL) and paw volume was reassessed every 10 minutes for 60 minutes post-5HT injection. Plethysmometry was conducted by an observer blind to the experimental condition.

Interstitial Fluid Collection and Enzyme-linked Immunosorbent Assay (ELISA)

A separate group of male rats, ovariectomized female rats, and cycling female rats (n = 6–7 per group) received a hindpaw injection of 200 μL CFA (1:1 in sterile saline; ipl) into the right hindpaw. Twenty-four hours later, rats were briefly gas anesthetized and rapidly decapitated. Inflamed rat hindpaw samples were collected with three 6 mm biopsy punches (Miltex, Inc., York, PA). Tissue biopsies were placed on a filter (5 mL polysterene round-bottomed tube with cell-strainer cap; BD Falcon, Franklin Lakes, NJ) and samples were centrifuged at 275 *g* for 20 minutes at 4°C to recover the interstitial fluid. A 1% ascorbic acid was then added to the collected fluid and was stored at -20°C. Interstitial fluid samples were then assayed in duplicate by a rat-specific Serotonin (Research) ELISA kit (IB89540; IBL Laboratories; Minneapolis, MN) and duplicate readings were averaged for analysis. The experimental design during preliminary analyses included saline-injected controls, however hindpaw interstitial fluid volume and 5HT content were below detectable levels of the assay so these groups were discontinued and statistical analyses were restricted to comparisons across sex and stage of cycle.

Data Analysis

Behavioral data were expressed as mean ± standard error of the mean paw withdrawal latencies in seconds or the force to withdraw in grams. Behavioral data and plethysmometry were analyzed by repeated measures analysis of variance (ANOVA) with time as the repeated factor and both stage (males, OVX, diestrus 1, diestrus 2, proestrus, estrus) and treatment (5HT, saline) as independent factors with statistical significance set at $p \leq 0.05$. Bonferonni's correction was used to calculate *a priori* pairwise comparisons with significance set at $p \leq 0.01$. Since sphericity was violated in the plethysmometry experiment, the Greenhouse-Geisser statistic was used for reporting multivariate statistics [40]. ELISA data were expressed as mean ± standard error of the mean ng/mL and analyzed by ordinary one-way ANOVA. Individual groups were compared using Bonferroni *post hoc* analysis. Statistical significance was tested at $p \leq 0.05$. Repeated measures multivariate data were analyzed using IBM SPSS Statistics version 25 (IBM, Armonk, NY) and all other data were analyzed using GraphPad software version 7 (GraphPad, San Diego, CA).

Results

Peripheral 5HT evokes significant thermal hyperalgesia in male rats, ovariectomized female rats, and female rats at each stage of the estrous cycle

We first tested whether intraplantar 5HT evoked significant thermal hyperalgesia across the estrous cycle in female rats, in males, and in ovariectomized females over a 30-minute time period. Thus, a 6 (stage) x 4 (time) x 2 (treatment) ANOVA was conducted. There was a significant three way interaction of time by treatment by stage [$F(14.569,74) = 2.124$; $p \leq 0.05$] and a two way interaction of time by treatment [$F(2.914,74) = 61.473$; $p \leq 0.05$]. The significant three-way interaction indicated that rats in different stages differed across time and between experimental and control groups. Two-way interactions and main effects were not interpreted due

to the presence of a significant three-way interaction term. Compared to saline controls, peripheral 5HT evoked significant thermal hyperalgesia at 10 minutes in males (see Figure 2.1A), ovariectomized females (see Figure 2.1B), and females at each stage of the estrous cycle (see Figure 2.1C-F; both $p \leq 0.01$). Only for proestrus and estrus was there significant hyperalgesia 20 minutes post-5HT injection (both $p \leq 0.01$), with sensitivity returning to levels comparable to saline controls in all groups by 30 minutes post-5HT injection (see Figure 2.1E-F; $p > 0.01$).

Peripheral 5HT evokes greater, longer lasting thermal hyperalgesia in female rats during proestrus and estrus when compared to males, ovariectomized females, and females in diestrus

We then tested whether 5HT-evoked thermal hyperalgesia was sexually dimorphic and dependent on fluctuations in the estrous cycle using a 6 (stage) x 4 (time) ANOVA. There was a significant between-subjects main effect of treatment [$F(1,74) = 53.027$; $p \leq 0.05$], stage [$F(5,74) = 10.284$; $p \leq 0.05$], and a significant treatment by stage interaction [$F(5,74) = 4.606$; $p \leq 0.05$]. Results of post hoc pairwise comparisons using a Bonferroni correction indicated that as early as 10 min post 5HT-injection, thermal hyperalgesia was significantly greater in females in estrus and proestrus compared to females in diestrus (both $p \leq 0.01$). At 20 minutes, 5HT-evoked thermal hyperalgesia to a greater degree in female rats in proestrus and estrus compared to all other groups (see Figure 2.1G; all $p \leq 0.01$). At 30 minutes, paw withdrawal latencies were comparable between all groups ($p > 0.01$). There were no differences in basal thermal sensitivity (0 time point; $p > 0.01$) or between females in proestrus and estrus at any time point ($p > 0.01$). There were also no differences between saline treated groups at any time points ($p > 0.01$). When paw withdrawal latencies of 5HT-injected females from all stages of the estrous cycle were collapsed and compared to males or ovariectomized females the sex difference was no longer observed (see Figure 2.1H; $F(6,126)=4.58$; $p > 0.05$), illustrating the importance of stratifying data in females by stage of the estrous cycle to detect a potentially masked sex difference.

Peripheral 5HT evokes significant mechanical allodynia in female rats during proestrus and estrus, but not during diestrus, following ovariectomy, or in male rats

We next tested whether intraplantar 5HT evoked significant mechanical allodynia across each stage of the estrous cycle in female rats, in intact males, and in ovariectomized females over a 30-minute time period. Thus, a 6 (rat stage) x 4 (time) x 2 (treatment) ANOVA was conducted. There was not a three-way interaction between time by treatment by stage [$F(14.361,73) = 0.601$; $p > 0.05$] or a time by stage interaction [$F(14.361,73) = 1.080$; $p > 0.05$], however there was a significant time by treatment interaction [$F(2.872,73) = 11.299$; $p \leq 0.05$] and a significant within-subjects main effect of time [$F(2.872,73) = 49.492$; $p \leq 0.05$]. The two-way interaction indicated treatment groups differed over time and treatment. Peripheral 5HT did not significantly alter mechanical sensitivity compared to saline injection in males (see Figure 2.2A), ovariectomized females (see Figure 2.2B), or females in diestrus 1 (see Figure 2.2C) or diestrus 2 (see Figure 2.2D; all $p \leq 0.01$). In female rats in proestrus (see Figure 2.2E) and estrus (see Figure 2.2F), 5HT evoked significant mechanical allodynia at 10 minutes post-injection (both $p \leq 0.01$). There were no differences in between-groups comparisons in saline or 5HT treated groups at any time points (see Figure 2.2G; $p > 0.05$).

5HT edema and 5HT content in interstitial fluid during inflammation of the rat hindpaw is comparable between male and female rats

We then tested whether intraplantar 5HT evoked significant hindpaw edema across the estrous cycle in female rats, in males, and in ovariectomized females. Thus, a 5 (stage) x 6 (time) x 2 (treatment) ANOVA was conducted. There was no significant three-way interaction of time by treatment by stage [$F(15.582,50) = 1.307$; $p > 0.05$] nor was there a significant time by stage interaction [$F(15.582,50) = 60.479$; $p > 0.05$]. There was a significant time by treatment interaction [$F(3.895,50) = 6.418$; $p \leq 0.05$] and a within-subjects significant main effect of time

[F(3.895,50) = 2.771; $p \leq 0.05$]. Peripheral 5HT evoked significant edema in males (see Figure 2.3A), ovariectomized females (see Figure 2.3B), and females in diestrus (see Figure 2.3C), proestrus (see Figure 2.3D), and estrus (see Figure 2.3E) when compared to saline vehicle controls at all time points tested (all $p \leq 0.01$). There were no differences in between-groups comparisons in saline or 5HT treated groups at any time points (see Figure 2.3F; $p > 0.05$).

We then tested whether inflammation evoked by CFA triggered differential levels of peripheral 5HT in male and female rats. Twenty-four hours of CFA-evoked inflammation in the rat hindpaw resulted in comparable interstitial levels of 5HT (see Figure 2.4; F(3,22) = 0.68; $p > 0.05$). The interstitial fluid of the inflamed hindpaw of male rats and ovariectomized females was 57.6 ± 1.3 ng/mL and 58.8 ± 1.3 ng/mL, respectively. The interstitial fluid of the inflamed hindpaw of female rats in diestrus was 55.5 ± 2.3 ng/mL, while local 5HT was at 60 ± 1.8 ng/mL in females in estrus and 59.9 ± 2.4 ng/mL in females in proestrus.

5HT-evoked thermal hyperalgesia and edema are blocked by antagonism of the excitatory $5HT_{2A}$ receptor subtype in both male and female rats

We next determined whether the $5HT_{2A}$ receptor subtype was a target for 5HT evoked-thermal hyperalgesia and edema in male and female rats. Local pretreatment with the selective $5HT_{2A}$ receptor antagonist M100907 15 minutes prior to 5HT injection blocked 5HT-evoked thermal hyperalgesia in male rats (see Figure 2.5A; F(1,8) = 16.30; $p \leq 0.004$), female rats in diestrus 1 or diestrus 2 (see Figure 2.5B; F(1,10)=6.08; $p \leq 0.03$), and female rats in proestrus or estrus (see Figure 2.5C; F(2,16) = 72.96; $p \leq 0.0001$). Vehicle-treated controls in proestrus and estrus displayed greater thermal hyperalgesia than males and females in diestrus 1 or diestrus 2 [F(2,14) = 4.78; $p \leq 0.03$], concurring with findings reported in Figure 2.1. A contralateral injection of M100907 into the left hindpaw prior to receiving an injection of 5HT in the right hindpaw did not block 5HT-evoked thermal hyperalgesia (see Figure 2.5C; $p > 0.05$). We then

tested whether local antagonism of the $5HT_{2A}$ receptor would also block 5HT-evoked hindpaw edema. Intraplantar injection of M100907 15 minutes prior to 5HT injection significantly attenuated 5HT-evoked edema in male rats (see Figure 2.6A; $F(1,10) = 77.30$; $p \leq 0.0001$) and female rats in proestrus and estrus (see Figure 2.6B; $F(2,19) = 26.48$; $p \leq 0.0001$).

Discussion

Understanding the pain mechanisms underlying the significantly greater prevalence of some persistent pain conditions in women is vital for improving women's health. In our previous research, we reported that 5HT in the male rat peripheral nervous system triggers pain via $5HT_{2A}$ and $5HT_3$ receptors and enhances pain signaling via the TRPV1 population of nociceptors [19, 21]. We then reported that 5HT triggers greater capsaicin-evoked proinflammatory peptide release from human dental pulp extracted during the luteal phase of the menstrual cycle, while no effect was observed in dental pulp extracted from males [41]. These findings led us to the hypothesis that hormones may be modulating this peripheral serotonergic pain mechanism in females. As no studies had yet injected 5HT peripherally into female rats, we designed a set of experiments to be the first to characterize the effects of peripheral 5HT on pain behaviors and edema in female rats across the estrous cycle. Here we report in that (1) peripheral 5HT evokes thermal hyperalgesia and edema in female rats at each stage of the cycle and mechanical allodynia only in proestrus and estrus females, (2) 5HT-evoked thermal hyperalgesia is sexually dimorphic and dependent on the estrous cycle, and (3) 5HT acts via, at least, the $5HT_{2A}$ receptor to trigger thermal hyperalgesia and edema in female rats.

Peripheral 5HT levels are positively correlated with pain in humans [42-44] and exogenous 5HT can induce local pain in humans [45-47]. In support, 5HT excites feline [48] and rodent [21, 49-51] nociceptors and injection of 5HT into the male rat hindpaw evokes significant hyperalgesia, largely through the excitatory $5HT_{2A}$ and $5HT_3$ receptors [12, 18, 19, 52, 53]

expressed on nociceptors. In the present study, we concur with these findings of a rapid and transient (~10 min) 5HT-induced hyperalgesia in male rats and we additionally report 5HT-evoked thermal hyperalgesia in ovariectomized rats and cycling female rats at each stage of the estrous cycle. Interestingly, female rats in proestrus and estrus remained hyperalgesic for 20 minutes that was greater than all other groups tested at the 20 min time point. While we did not observe significant 5HT-evoked mechanical allodynia in male rats, ovariectomized females, or females in diestrus, we did observe significant mechanical allodynia in females in proestrus and estrus. Together these data indicate that females with fluctuating gonadal hormone levels may experience greater and prolonged peripheral 5HT-evoked pain. It was interesting that males, ovariectomized females, and females in diestrus displayed 5HT-evoked thermal hyperalgesia, but not mechanical allodynia. As we designed our experiments utilizing a low dose of 5HT (2 μg / 100 μL) to retain physiological relevance to 5HT released during injury states [18, 53-55], it may be that this dose of 5HT is not sufficient to trigger allodynia in rats in low estrogen states, but is sufficient to trigger hyperalgesia in all groups and to a greater degree in rats in high estrogen states, indicating a pronociceptive role of estrogen on 5HT-evoked pain. Alternatively, it may be that serotonergic potentiation of TRPV1 may be critical for thermal hyperalgesia but not mechanical allodynia at this concentration.

Of special note, when all estrous cycle groups were collapsed into the same group and compared to males and ovariectomized females for analysis, the sex difference in 5HT-evoked thermal hyperalgesia was masked. These data clearly illustrate the importance of stratifying data by the stage of the estrous cycle, especially in cases where the pain mechanism is present or exacerbated during only one or two stages of the 4-day estrous cycle. In support, in our previous study, we examined 5HT-evoked thermal hyperalgesia in males, ovariectomized females, and cycling females and did not observe a sex difference in 5HT-evoked thermal hyperalgesia [19].

As estrogen levels are low in ovariectomized females and females in diestrus (~15–20 pg/mL), then peak and rapidly decline during proestrus and estrus (~40–60 pg/mL) [37, 56, 57], our data reporting 5HT-evoked thermal hyperalgesia and mechanical allodynia in females during proestrus and estrus implicate a role of estrogen in 5HT-evoked pain.

We designed these studies utilizing naturally cycling female rats under the hypothesis that estrous cycle differences in pain behaviors will be more likely detected during periods of hormone fluctuations, rather than steady-state hormone levels achieved by ovariectomy and hormone replacement with hormone implants. This is supported by the report that nociception and morphine analgesia are not altered during steady-state hormone dosing [56] despite many reports of sex differences and estrous-cycle effects on pain and morphine analgesia [36, 58-65]. Indeed, we observed a significant effect of estrous cycle in our female rats, which might be attributed to this serotonergic pain mechanism being altered during rising (proestrus) *and* falling (estrus) levels of estrogen. Alternatively, rising levels of estrogen during proestrus may trigger this serotonergic pain mechanism, which may remain active during estrus, when estrogen levels are falling. While we know that 5HT is acting via TRPV1 nociceptors to trigger and enhance pain in males, it remains unclear whether this is true of females and how estrogen is altering this serotonergic pain mechanism. Future experiments are warranted to determine specific genomic and non-genomic mechanisms estrogen is acting on to alter this serotonergic pain mechanism in females. A likely candidate is the G protein-coupled estrogen receptor GPR30. Lu et al. reported that 5-hydroxytroptophan-induced visceral sensitivity was eliminated following ovariectomy, but rapidly restored with an ERα agonist and a GPR30 agonist, but not an ERß agonist [66].

It is unknown whether estrogen is altering local 5HT levels or changes in the nociceptors. Estrogen has been reported to increase serum 5HT levels and 5HT synthesis in the central nervous system [67-69], but it remains unknown if this also occurs in the periphery. Additionally,

estrogen treatment has been shown to slow clearance of 5HT in the brain via blocking SERT, the serotonin transporter [70, 71]. A major source of peripherally released 5HT during injury are the immune cells, which can both synthesize 5HT and express SERT which can uptake 5HT made by enterochromaffin cells [72]. Sex differences in 5HT content in immune cells have also been reported [73]. It may be possible that estrogen is altering SERT function in immune cells, which may account for the prolonged 5HT-induced pain behaviors observed in females in the proestrus into estrus stages. In support, SERT knockout mice display reduced thermal hyperalgesia [74] correlated to an increase in 5HT levels at the site of injury [75]. Progesterone can also block SERT function and levels are also low in ovariectomized females and females in diestrus and rapidly rise and decline during proestrus and estrus; however progesterone is known to be anti-inflammatory and anti-nociceptive [76-80] so it is not likely to be involved in this observed sex difference. While there were no sex differences or estrous cycle effects of 5HT on edema or 5HT content in interstitial fluid from the inflamed hindpaw in the present study, it is likely that our observed sex difference and estrous cycle effects are due to direct excitatory actions of 5HT on nociceptors. A limitation of our analysis is that 5HT content in saline-injected control animals was below the detectable levels of the assay, hence statistical analysis is restricted to comparisons across sex and stage of cycle. Future studies utilizing more sensitive methodology, such as microdialysis, are required to perform analysis on 5HT levels in naïve versus inflamed animals.

For 5HT to have direct actions on nociceptors, 5HT would need to bind to excitatory 5HT receptors localized on nociceptors. 5HT receptors are expressed on nociceptors, including TRPV1-positive nociceptors [21, 50], and some subtypes may be able to sensitize TRPV1 via altering the ion channel's phosphorylation through metabotropic 5HT receptor signaling cascades. Of the excitatory receptor subtypes, which include $5HT_2$, $5HT_3$, $5HT_4$, $5HT_6$, and $5HT_7$, the $5HT_{2A}$ and $5HT_3$ are reportedly expressed in the peripheral nervous system [81-83], including

on TRPV1-positive nociceptors [21, 50]. The $5HT_3$ receptor subtype is an excitatory 5HT-gated ion channel that may directly alter membrane excitability in the central and peripheral nervous system to increase pain [50, 84, 85]. The $5HT_{2A}$ subtype is coupled to excitatory G protein activation of PKC, which has been shown to sensitize TRPV1 via phosphorylation [13] and increase pain [17, 18]. Since we previously reported that in male rats, activation of the $5HT_{2A}$ receptor enhances capsaicin-evoked CGRP release and capsaicin-evoked pain [19, 21], we tested whether this was true of 5HT-evoked pain in females. Local injection with the selective $5HT_{2A}$ receptor antagonist, M100907, prior to intraplantar 5HT injection blocked thermal hyperalgesia in both males and females across the estrous cycle. Injection of the antagonist into the contralateral hindpaw did not block 5HT-evoked thermal hyperalgesia; this indicates that 5HT is acting at peripheral $5HT_{2A}$ receptors on nociceptors to trigger pain in both male and female rats.

Local administration of M100907 also attenuated 5HT-evoked edema in male and female rats, which was not observed when the antagonist was administered contralateral. Together these data suggest that 5HT acts via peripheral $5HT_{2A}$ receptors to trigger pain and edema in both males and females across the estrous cycle. Interestingly, it can be observed that one injection of 5HT evokes similar edema in both males and females at 10 and 20 minutes post-injection (~35–45% in males and 45–55% in females; Figure 2.3), while an injection of vehicle prior to injection with 5HT leads to an apparent greater edema in males at the 10 and 20 minute time points (~65–75% in males and ~55% in females; Figure 2.6). While anecdotal under this present experimental design, this is an interesting observation that warrants future examination of potential sex differences in immune responses to multiple insults.

Overall, our study indicates that 5HT evokes sex-specific and estrous cycle dependent effects on pain. Peripheral 5HT evokes thermal hyperalgesia in both males and females, and to a greater degree in females in proestrus and estrus, via the $5HT_{2A}$ receptor and evokes mechanical

allodynia only in females in proestrus and estrus. Further, 5HT-evoked thermal hyperalgesia lasted twice as long in females in proestrus and estrus compared to males, OVX females and females in diestrus, implicating a role of estrogen modulation. While 5HT-evoked edema and local 5HT levels in the interstitial fluid following an inflammatory insult in the hindpaw were not significantly different between males and females or across the estrous cycle, it is likely that our observed sex difference and estrous cycle effects are due to differences in the effects of estrogen on 5HT-evoked pain. Future studies elucidating the mechanisms underlying estrogen modulation of 5HT-evoked pain and examination of the role of 5HT in pain conditions that model clinically-relevant pain disorders more common in women is warranted by our data. Gaining insight on female-specific pain mechanisms has the potential to improve the clinical management of the various pain disorders observed predominately or only in women.

Glossary

Thermal Hyperalgesia: heightened sensitivity to a noxious thermal stimulus.

Mechanical Allodynia: developed sensitivity to an innocuous stimulus.

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Figures

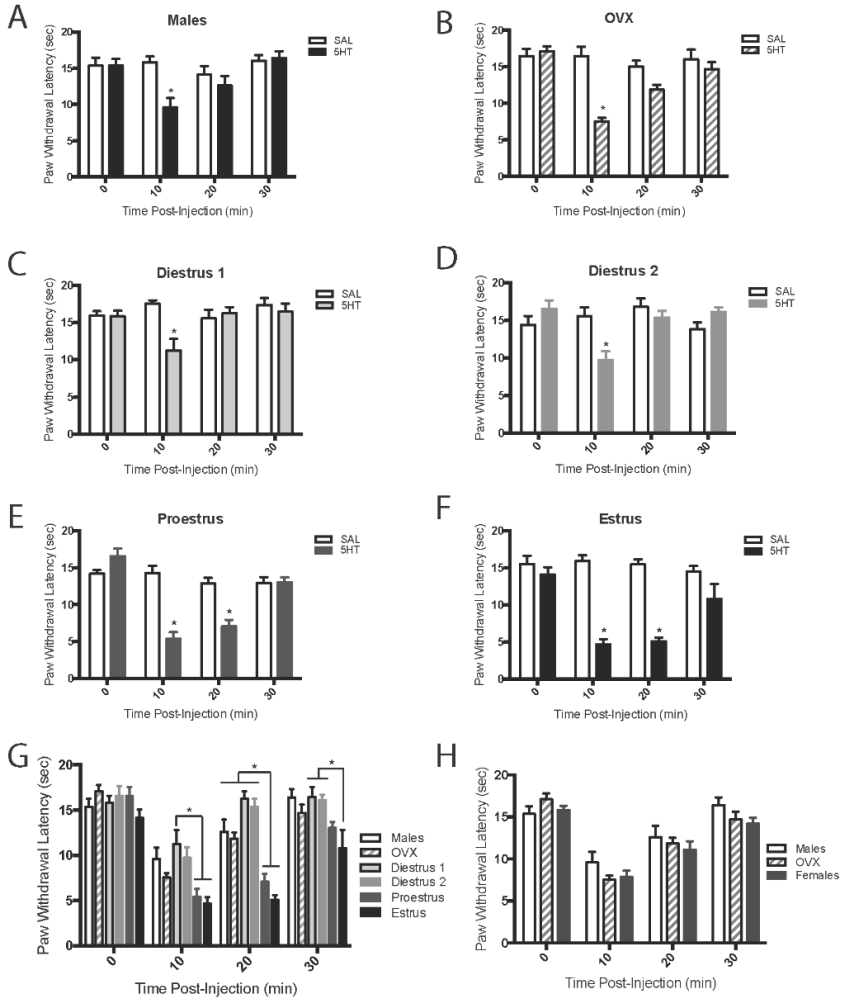

Figure 2.1. Peripheral 5HT evoked greater and long-lasting thermal hyperalgesia in female rats in proestrus and estrus. Serotonin (5HT; closed bars) evokes significant acute thermal hyperalgesia observed as a decrease in paw withdrawal latency in male rats (A), ovariectomized females (B), and females in diestrus 1 (C), diestrus 2 (D), proestrus (E), and estrus (F) compared to saline vehicle controls (SAL; open bars). * denotes a significant effect of 5HT compared to saline per time point with significance in pairwise comparisons tested at $p \leq 0.01$. 5HT evokes significantly greater thermal hyperalgesia during proestrus and estrus at 10 minutes post-injection when compared to females in diestrus 1 and at 20 mins post-injection when compared to all other groups (G). * denotes a significant difference in paw withdrawal latency between sex/stage during a single time point. Significance in pairwise comparisons were tested at $p \leq 0.01$. When the analysis was conducted with the cycling female experimental groups collapsed and compared to males and ovariectomized females, a significant difference was no longer observed (H).

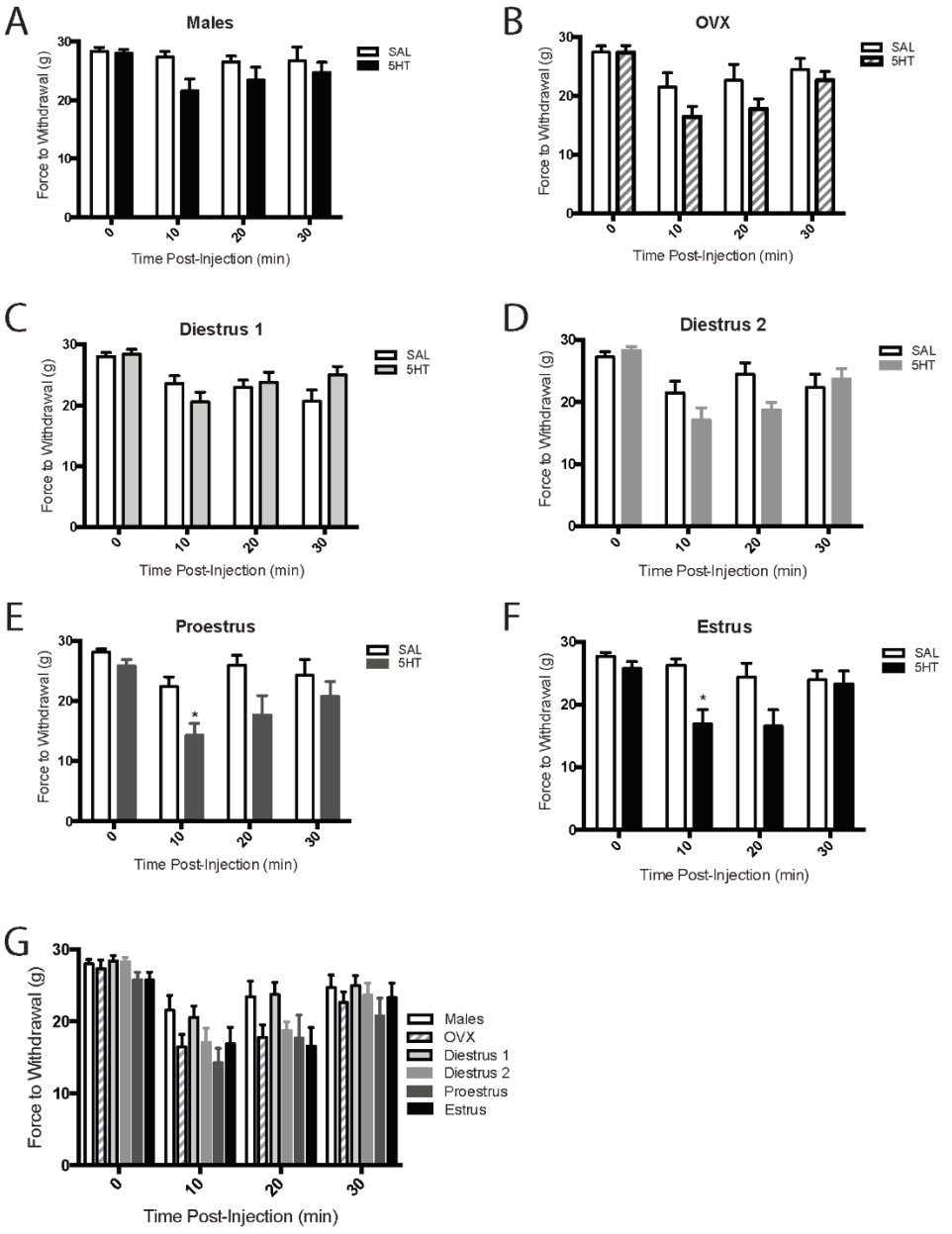

Figure 2.2. Peripheral 5HT evoked mechanical allodynia in female rats in proestrus and estrus only. Serotonin (5HT; closed bars) did not evoke mechanical allodynia in male rats (A), ovariectomized females (B), or females in diestrus 1 (C) or diestrus 2 (D) compared to saline vehicle controls (SAL; open bars). 5HT did evoke significant mechanical allodynia observed as a decrease in the grams of pressure required to elicit paw withdraw in female rats in proestrus (E) and estrus (F). * denotes a significant effect of 5HT compared to saline per time point with significance in pairwise comparisons tested at $p \leq 0.01$. No significant differences were observed when force to withdraw was compared between groups at each time point (G).

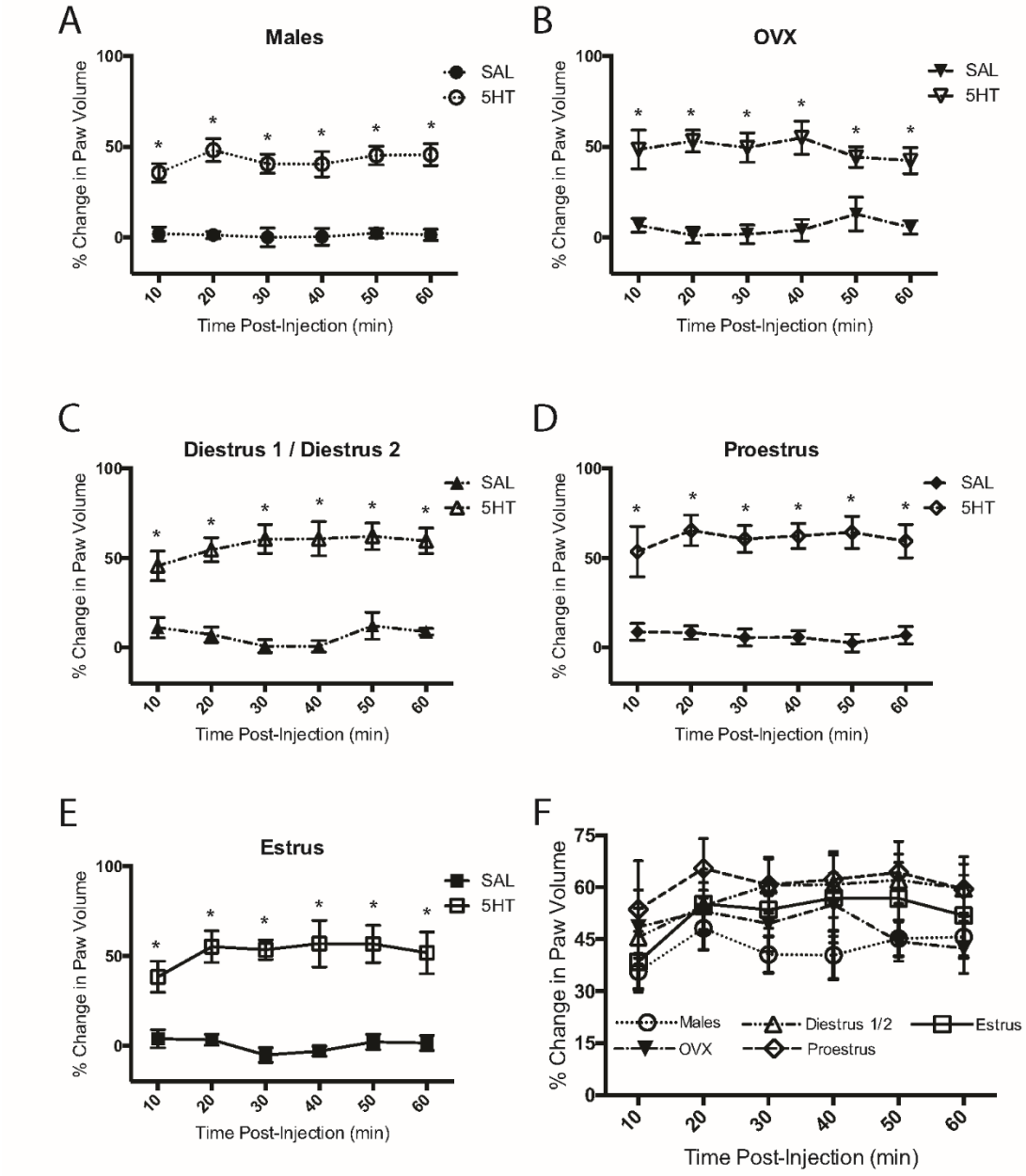

Figure 2.3. No differences in 5HT-evoked edema. Serotonin (5HT; open symbols) evoked significant edema observed as percent change in paw volume following intraplantar injection in male rats (A), ovariectomized females (B), and females in diestrus (stages 1 and 2 combined, C), proestrus (D), and estrus (E) when compared to saline vehicle controls (SAL; closed symbols). * denotes a significant difference between treatment per time point with significance tested at $p \leq 0.05$. 5HT does not evoke sexually dimorphic or estrous cycle dependent changes in edema (F).

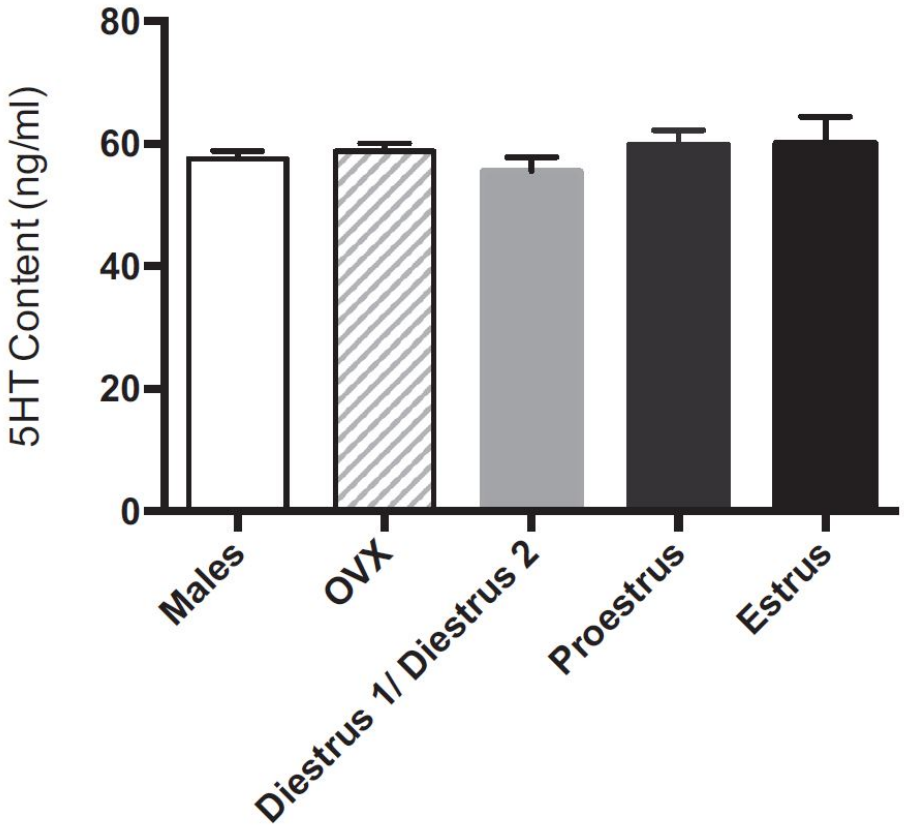

Figure 2.4. No differences in 5HT content in interstitial fluid from inflamed hindpaws. Twenty-four hours of CFA-evoked inflammation in the hindpaw resulted in comparable levels of 5HT in the hindpaw interstitial fluid of male rats (white), ovariectomized females (striped), and females in diestrus (stages 1 and 2 combined; light grey), proestrus (dark grey), and estrus (black).

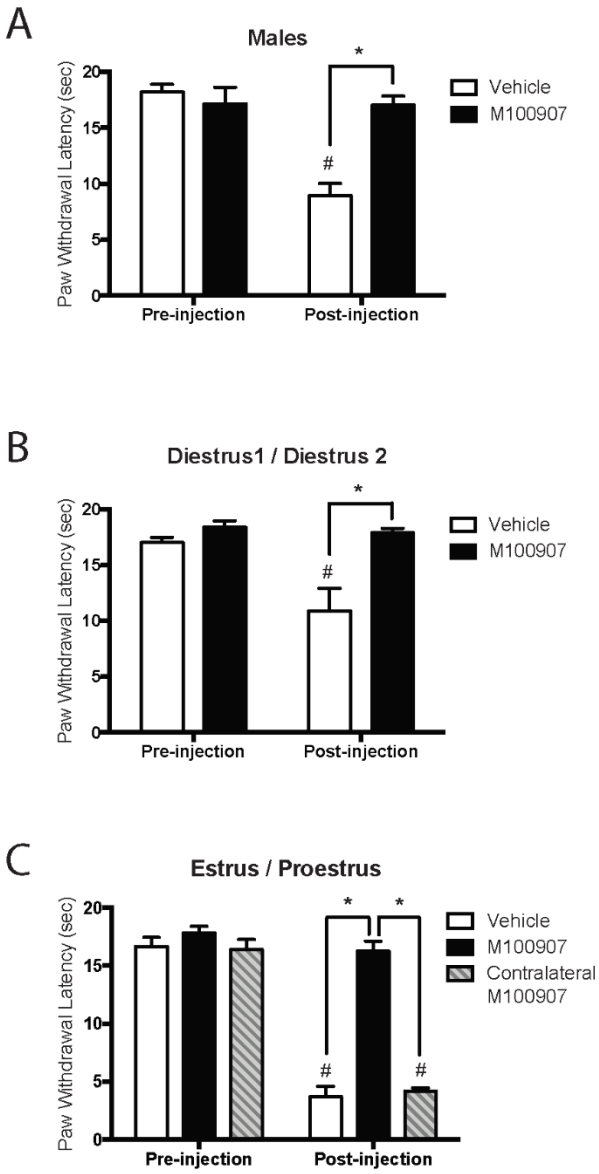

Figure 2.5. $5HT_{2A}$ antagonist blocked thermal hyperalgesia in male and female rats. Pretreatment with the selective $5HT_{2A}$ antagonist, M100907, (black bars) blocked serotonin-evoked thermal hyperalgesia in male rats (A), and females in diestrus 1 or diestrus 2 (B) and proestrus or estrus (C) compared to vehicle-pretreated controls (open bars). Female rats in proestrus or estrus that received a left hindpaw injection of M100907 contralateral to serotonin injection in the right hindpaw remained hyperalgesic (C; lined bars). * denotes a significant difference between treatment groups. # denotes a significant difference between pre- and post-injection. Significance was tested at $p \leq 0.05$.

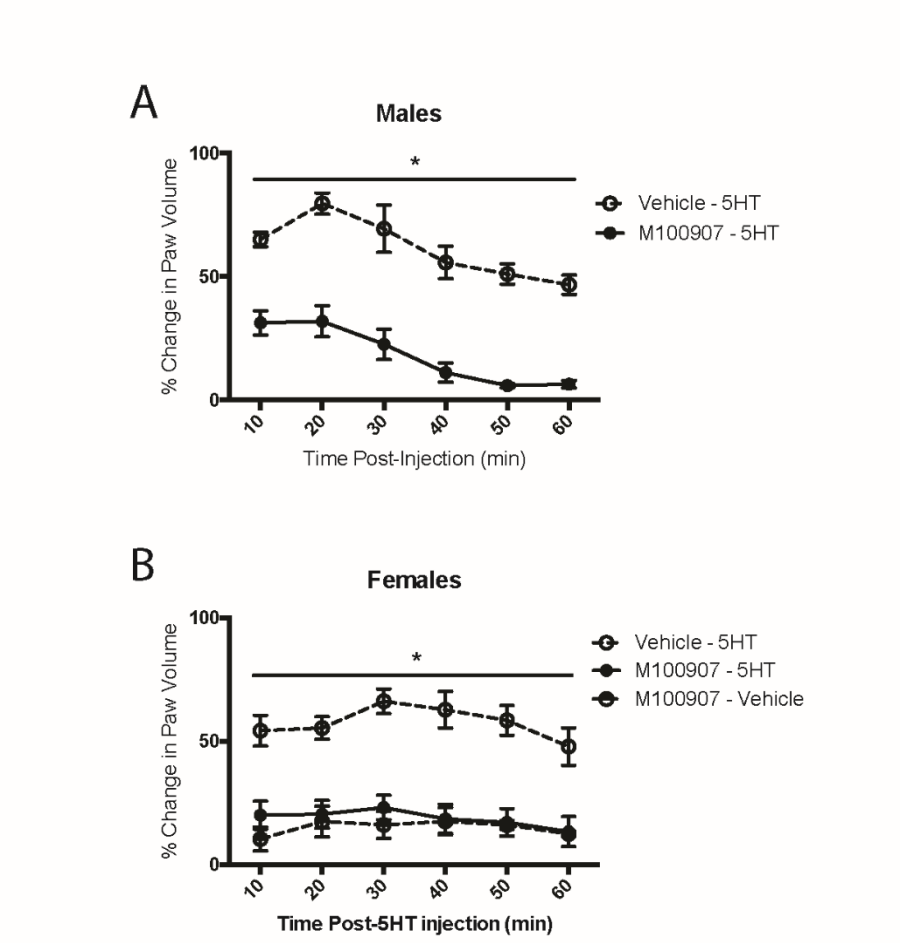

Figure 2.6. $5HT_{2A}$ antagonist attenuated 5HT-evoked edema in male and female rats. Local pretreatment with the selective $5HT_{2A}$ antagonist, M100907, prior to 5HT injection (closed circles) attenuated serotonin-evoked edema in male rats (A) and female rats in proestrus or estrus (B) as compared to vehicle controls (open circles). Treatment with M100907 prior to vehicle injection did not have an effect on edema (B; partial closed circles). * denotes a significant difference between treatment groups per time point. Significance was tested at $p \leq 0.05$.

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CHAPTER III

ESTROGEN EXACERBATES THE NOCICEPTIVE EFFECTS OF PERIPHERAL SEROTONIN ON RAT TRIGEMINAL SENSORY NEURONS

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Short running title: E2 and 5HT modulate trigeminal pain processing

Abstract

Orofacial pain disorders involving trigeminal sensory neurons disproportionately affect women and can be modulated by hormones, especially estrogen (E2). Proinflammatory mediators, like serotonin (5HT), can act on sensory neurons expressing the transient receptor potential vanilloid 1 (TRPV1) ion channel, resulting in peripheral sensitization. We previously reported peripheral 5HT evokes greater pain behaviors in the hindpaw of female rats during proestrus and estrus, stages when E2 fluctuates. It is unknown if this interaction is comparable in the trigeminal system. We hypothesized that *E2 exacerbates 5HT-evoked nocifensive pain behaviors and pain signaling in female trigeminal sensory neurons.* We report 5HT-evoked nocifensive behaviors are significantly higher during estrus and proestrus, which is attenuated by blocking the $5HT_{2A}$ receptor. The comparable dose of 5HT was not nociceptive in males unless capsaicin was also administered. When administered with capsaicin, a lower dose of 5HT evoked trigeminal pain behaviors in females during proestrus. Further, basal 5HT content in the vibrissal pad was higher in cycling females compared to males. *In vitro*, E2 enhanced 5HT-potentiated CGRP release from trigeminal neurons, which does not appear to occur via the $5HT_{2A}$ receptor. Our data indicates that estrogen fluctuation influences the pronociceptive effects of 5HT on trigeminal sensory neurons.

Keywords: Estrogen, Serotonin, Pain, $5HT_{2A}$ receptor, Trigeminal Sensory Neurons

Introduction

Orofacial pain is defined as "pain whose origin is below the orbito-meatal line, above the neck and anterior to the ears, including pain within the mouth" (Zakrzewska & Hamlyn, 1999) and includes pain disorders such as burning mouth syndrome, temporomandibular joint disorder (TMD), fibromyalgia and trigeminal neuralgia. According to the National Institute of Dental and Craniofacial Research, these pain conditions affect approximately 5–12% of the population, costs $4 billion annually, and develop into chronic pain conditions for 15% of patients. Moreover, orofacial pain disorders disproportionately affect women, with TMD and migraine being at least 3 times more common in women (Buse et al., 2013; LeResche, 1997; Wolfe et al., 1995). Also, according to epidemiological studies, pain-related symptoms are more severe, more common, and longer lasting in females (Buse et al., 2013; Fillingim, 2000). Interestingly, many orofacial pain disorders in women worsen in the luteal and menstrual phases of the menstrual cycle when gonadal hormones, estrogen and progesterone, greatly fluctuate and pain is often relieved during pregnancy and menopause (Fejes-Szabo et al., 2018; Marcondes et al., 2002). These clinical reports implicate a modulatory role of gonadal hormones on the sensory system innervating the orofacial region.

While the sensory neurons of the dorsal root ganglia (DRG) innervate the trunk and extremities, the sensory neurons of the trigeminal ganglia (TG) innervate the cranial and orofacial tissues. A subpopulation of trigeminal neurons expresses the Transient Receptor Potential Vanilloid 1 (TRPV1) ion channel, a nociceptor activated by diverse noxious stimuli, including noxious heat (> 42°C), capsaicin, protons, and endogenous lipids; resulting in calcium influx in the cell and release of calcitonin gene related peptide (CGRP), thus initiating nociceptive signaling (Basbaum et al., 2009; Jeske et al., 2008; Ruparel et al., 2012). Activation and sensitization of trigeminal nociceptors underlying orofacial pain involves recruitment of immune

cells such as mast cells, platelets, and macrophages (Ji et al., 2016; Shinoda et al., 2019). These immune cells release an acidic milieu of proinflammatory and pronociceptive mediators, such as bradykinin, histamine, prostaglandins, and serotonin (5HT). These mediators activate signaling cascades in sensory neurons to contribute to peripheral sensitization, thus reducing the threshold for activation of nociceptors which underlies an increase in pain sensitivity and may ultimately contribute to the development of chronic pain conditions.

In the periphery, 5HT is stored and produced by injured epithelial cells, gut enterochromaffin cells, macrophages, T cells, mast cells, and platelets (Herr et al., 2017; Mössner & Lesch, 1998; Ni et al., 2008; Spohn & Mawe, 2017). 5HT is a major pro-inflammatory molecule that can act through seven known subtypes of 5HT receptors ($5HT_1$-$5HT_7$) to induce pain. Previous studies in male rats have shown that excitatory $5HT_{2A}$ and $5HT_3$ receptor subtypes are localized to trigeminal sensory neurons that also express TRPV1, leading to an enhanced capsaicin-evoked Ca^{+2} influx and CGRP release, that is attenuated in presence of $5HT_{2A}$ and $5HT_3$ receptor antagonists (Loyd, Chen, et al., 2012; Loyd et al., 2011). 5HT also potentiates capsaicin-evoked CGRP release in human dental pulp isolated from females during luteal phase of the menstrual cycle (Loyd, Sun, et al., 2012). Similarly, 5HT injection into the masseter muscle of healthy human females leads to increased hyperalgesia and allodynia, which is abolished in presence of $5HT_3$ antagonist, granisetron (Ernberg et al., 2000). In support, our lab has reported that intraplantar injection of 5HT evokes greater and longer lasting pain behaviors in female rats during proestrus and estrus compared to males, diestrus, and ovariectomized females and these pain behaviors are attenuated in the presence of the $5HT_{2A}$ antagonist, M100907 (Kaur et al., 2018). We have also reported that blocking the $5HT_3$ receptor with granisetron does not attenuate 5HT-evoked orofacial pain behaviors in male and female rats (Kaur et al., 2021a). This

suggests a potential neuromodulatory role of $5HT_{2A}$ receptors in sex differences in trigeminal pain processing.

While progesterone plays a clear antinociceptive and anti-inflammatory role on pain processing, estrogen's (E2) role remains controversial with reports of both pronociceptive and antinociceptive effects. The pronociceptive role of E2 has been well documented in both animal and human models. In rats, a high concentration of E2 results in upregulation of TRPV1 and nerve growth factor (NGF) expression in the central and peripheral nervous system (Wu et al., 2015; Yamagata et al., 2016), enhances allodynia in the temporomandibular joint (Wu et al., 2015), and increases formalin-induced orofacial pain behavior (Fejes-Szabo et al., 2018). E2 also has pronociceptive effects *in vitro* on pain signaling. Specifically, E2 causes a rapid Ca^{+2} influx into the epithelial cells via TRPV6 channel (Irnaten et al., 2008), enhances bradykinin signaling in rat sensory neurons (Rowan et al., 2010), and regulates release of CGRP (Pota et al., 2017). On the contrary, high E2 has also been associated with analgesic effects in women with chronic pain (Hellstrom & Anderberg, 2003). In rats, E2 treatment has been reported to reduce nociceptive neural activity in cultured neurons and reduce nocifensive behaviors in ovariectomized (OVX) rats (Averitt et al., 2019). In any case, there is a clear association between trigeminal pain disorders and hormone status.

E2 may also modulate pain processing by influencing the levels of 5HT in the peripheral nervous system. It has been shown that externally administered E2 can maintain high levels of 5HT in the periphery in OVX rats (Benmansour et al., 2016). Also, E2 can increase the expression of tryptophan hydroxylase enzyme (TPH; rate limiting enzyme for 5HT synthesis), decrease the expression of serotonin reuptake transporter, and increases the binding and density of $5HT_{2A}$ receptors in the central nervous system (Akira Kugaya et al., 2003; Bethea et al., 2000;

Moses-Kolko et al., 2003). Additionally, 5HT-evoked CGRP release is highest from the dental pulp of females in the last week of menses (Loyd, Sun, et al., 2012).

Together, these studies suggest a possible neuromodulatory role of E2 on 5HT-evoked pain signaling in trigeminal sensory neurons. As orofacial pain disorders are more prevalent in women and there is evidence that E2 modulates pain and the serotonergic system, we focused our present work on determining whether there is an interaction between E2 and peripheral 5HT in trigeminal sensory neurons in a rat model of orofacial pain. We hypothesized that E2 exacerbates the nociceptive effects of 5HT on rat trigeminal sensory neurons.

Methods

Subjects

A total of 83 adult male and 313 adult female Sprague–Dawley rats (200–300 g; Charles River Laboratories, Wilmington, MA) were used in the experiments. Rats were separated by sex and pair-housed in a 12:12-h light: dark cycle with ad libitum food and water access. All studies were approved by the Texas Woman's University Institutional Animal Care and Use Committee and conform to federal guidelines and guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain. This study was conducted in strict compliance with the Animal Welfare Act, implementing Animal Welfare Regulations, and the principles of the Guide for the Care and Use of Laboratory Animals.

Vaginal cytology

Vaginal lavages were performed between 0900AM to 1100AM at 24-h intervals beginning 2 weeks (at least two consecutive cycles; 10 days) before testing to confirm that all female rats were cycling normally. Daily records were maintained on the stages of their cycle throughout experimental testing. Proestrus was identified as a predominance of nucleated epithelial cells and estrus was identified as a predominance of cornified epithelial cells. Diestrus 1

(or metestrus) was differentiated from diestrus 2 (or diestrus) by the presence of leukocytes (Becker et al., 2005; Loyd et al., 2008; McLean et al., 2012). When no significant differences were noted in behavior of diestrus 1 and diestrus 2 animals, these data were pooled and reported as such.

Ovariectomy

Female rats (n = 96) were deeply gas anesthetized (3% induction; 2.5% maintenance) by inhalation of Isothesia (isoflurane, USP, Henry Schein Animal Health, Dublin, OH) and a single incision was made across the abdomen. The abdominal muscle was opened and the ovary bundles were ligated with 4-O silk sutures, excised, and removed, as previously described (White & Uphouse, 2004). The fascia was closed with 5-O silk suture and the skin was closed with Vicryl sutures to prevent wicking. Rats were allowed 2 weeks for recovery and ovarian hormone dissipation.

Drugs

Serotonin hydrochloride (5HT; Sigma–Aldrich, St. Louis, MO) was dissolved in double-distilled water and diluted in 0.9% sterile saline or Hank's balanced salt solution (HBSS) buffer immediately prior to each use. Capsaicin (CAP; Sigma–Aldrich, St. Louis, MO) was dissolved in 100% ethanol in a fume hood and aliquots were stored at -20°C as 100 mM stocks. Capsaicin was freshly diluted in 0.9% saline or HBSS buffer prior to each use. The $5HT_{2A}$ antagonist, M100907 (Sigma–Aldrich), was dissolved in 20% dimethyl sulfoxide (DMSO) in 0.9% sterile saline, stored as a 2 mM stock solution at 4 °C, and then serial diluted the day of use to a final working solution of 2 nM or 30nM M100907 (6% DMSO in 0.9% sterile saline). β-Estradiol (E2; Sigma–Aldrich, St. Louis, MO) was dissolved in 100% ethanol to create a 10 mM stock solution that was further diluted in HBSS buffer for a working solution of 50 nM. Immediately prior to use, complete Freund's adjuvant (CFA; Sigma–Aldrich) was dissolved 1:1 in 0.9% sterile saline.

Orofacial Nocifensive Behavior Testing

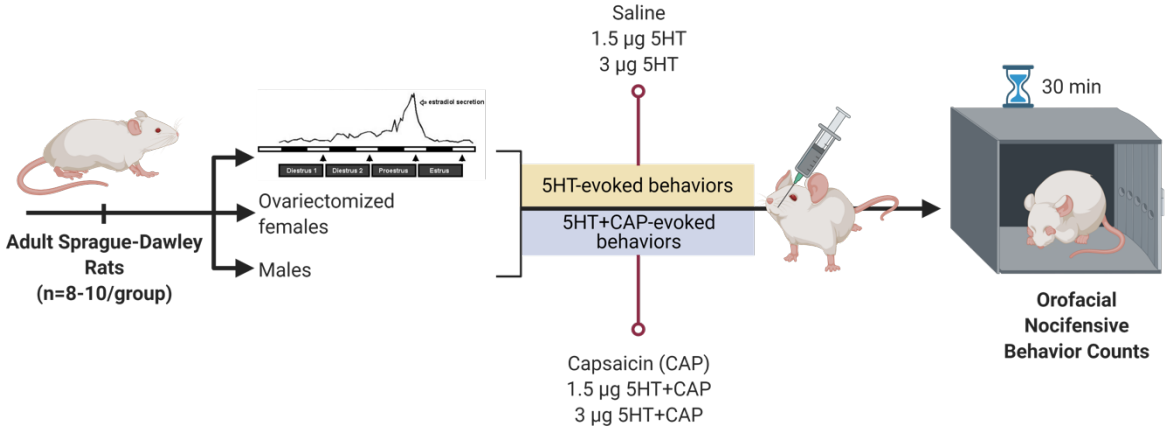
Square-shaped plexiglass boxes (30 x 30 x 30 cm) with mirrored sides (fabricated in-house) were used to observe orofacial nocifensive behaviors. Rats were acclimated to the behavior testing apparatus 24 hours prior to testing. On the day of testing, rats were placed in the individual boxes immediately post-injection and nocifensive behavior was recorded with a video camera for a 30-min time period. The videos were manually quantified using iMovie software (Apple Inc., Mac OS) by counting the number of forelimb swipes directed at the injection site in 6 min bouts over a 30 min period and reported as a measure of spontaneous nocifensive behavior. Rat forelimb swipes have been characterized in the literature as spontaneous nocifensive behavior distinct from grooming and itch behaviors (Shimada & LaMotte, 2008; Spradley et al., 2012). Data was collected by two independent observers blind to the experimental condition and their values were averaged. If there were substantial differences between the two observer's counts (> 5 swipes), they were re-evaluated together to concur for final reporting.

Adult male, cycling females at each stage of the estrous cycle, and ovariectomized (OVX) females (n = 9–11 per sex, stage of estrous cycle, and experimental treatment group) were gas anesthetized and received a single intradermal injection (30-gauge needle) of either 1.5 µg/50 µL 5HT, 3 µg/50 µL 5HT, or vehicle control (0.9% sterile saline; 50 µL) into the vibrissal pad (unilateral). The number of forelimb swipes over the injected area were counted as described above as 5HT-evoked nocifensive behaviors (see timeline). A separate group of rats received a single intradermal injection of each dose of 5HT + a low dose of capsaicin (1.5 µg 5HT + 1 µg CAP/50 µL; 3 µg 5HT + CAP/50 µL) or control (1 µg CAP/50 µL) into the vibrissal pad (unilateral). The number of forelimb swipes over the injected area were counted as described above as the effects of 5HT on capsaicin-evoked nocifensive behaviors (see Timeline 3.1). The dose of capsaicin was chosen based on a previous study characterizing the concentration-response

of injection of capsaicin into the rat vibrissal pad and a low dose was chosen to allow for observing a potential sensitizing effect of 5HT on capsaicin (Pelissier et al., 2002).

Timeline 3.1

Illustration of time course of groups and treatments for behavior studies. Created with BioRender.com.



A separate group of rats consisting of females in either proestrus or estrus and males (*n* = eight per sex and experimental treatment group) received an intradermal injection of the selective $5HT_{2A}$ antagonist, M100907 (2nM; 0.07 ng/100 µL) or vehicle control (100 µL 6% DMSO in 0.9% sterile saline) into the vibrissal pad (unilateral). Fifteen-minutes after the pre-treatment, female rats received an injection of 5HT (3 µg/50 µL) and the male rats received an injection of 5HT+CAP (3 µg 5HT + CAP/50 µL) at the same site. Orofacial nocifensive behavior was recorded and counted as described above. The time course and concentration of M100907 was chosen for selectivity based on previous studies reporting binding affinity (Ki = 1.92 nM) of M100907 for the $5HT_{2A}$ receptor (http://pdsp.med.unc.edu).

Interstitial fluid collection and enzyme-linked immunosorbent assay (ELISA)

A separate group of male rats, OVX female rats, and cycling female rats (*n* = 6–7 per group) received a left vibrissal pad injection of 50 µL CFA (1:1 in sterile saline) and right

vibrissal pad injection of vehicle control (50 μL 0.9% sterile saline). Twenty-four hours later, rats were briefly gas anesthetized and rapidly decapitated. Inflamed rat vibrissal pad samples were collected with four 6-mm biopsy punches (Miltex, Inc., York, PA). Tissue biopsies were placed on a filter of the cell-strainer tube (5 mL polystyrene round-bottomed tube with cell-strainer cap; BD Falcon, Franklin Lakes, NJ) and samples were centrifuged at 275 g for 20 min at 4 °C to recover the interstitial fluid. Then 1% ascorbic acid was then added to the collected fluid (to prevent 5HT degradation) and samples were stored at -20 °C. Interstitial fluid samples were then assayed in duplicate by a rat-specific Serotonin (Research) ELISA kit (IB89540; IBL Laboratories; Minneapolis, MN) and duplicate readings were averaged for analysis.

Primary culture of trigeminal ganglia neurons

Trigeminal ganglia (TG; n = 3–4 rats per 24-well plate run in triplicate) were extracted from adult OVX female rats (~200 g) immediately following decapitation. Primary neuron cultures were prepared using previously described methods (Loyd et al., 2011; Patwardhan et al., 2005). Briefly, TGs were suspended in HBSS on ice and gently washed three times. After dissociation with collagenase (5%, Worthington Biochemical Corp, Lakewood, NJ) and trypsin (1%, Sigma–Aldrich) at 37 °C, the cells were suspended in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Waltham, MA) containing 10% fetal bovine serum, glutamine, penicillin-streptomycin, nerve growth factor (NGF, 100ng/ml; Harlan, Indianapolis, IN), and treated with mitotic inhibitors (5-fluoro-2'-deoxyuridine and uridine). Cells were then lightly dissociated using a 20-gauge followed by a 23-gauge needle and then applied to 24-well poly-D-lysine-coated plates (Corning Inc., Corning, NY) and maintained in an incubator at 37 °C and 5% CO_2.

CGRP release assay

TG primary cultures were maintained for 5 days prior to running the CGRP release assay. The assay was performed using a protocol previously described (Loyd et al., 2011). Briefly,

cultures were washed with 300 µL HBSS twice to obtain baseline CGRP release. Cultures were then pretreated row-wise with either 5HT (100 µM), E2 (50 nM), M100907 (30nM) a combination of 5HT + E2, or HBSS followed by stimulation with CAP (50 nM). Each treatment lasted for 15 min. The concentration of each drug was chosen based on reported known binding affinities (http://pdsp.med.unc.edu). Superfusate was collected following each treatment and quantitated for CGRP levels by rat-specific CGRP ELISA (Cayman Chemical, Ann Arbor, MI). All experiments were conducted in duplicate with n = six wells per treatment group for a total of approximately 12 wells per group.

Data analysis

All data were analyzed with GraphPad Prism software version 8.3.0 (GraphPad, San Diego, CA) or IBM SPSS Statistics version 25 (Armonk, NY). All data graphs were made with GraphPad Prism. Orofacial nocifensive behavior data were expressed as mean ± standard error of the mean (SEM) number of forelimb swipes over a 30 min time period (6 min bouts). Behavior data was analyzed by repeated measures two-way and three-way analysis of variance (ANOVA) with time as the repeated factor and both stage (males, OVX, diestrus 1 and 2 combined, proestrus, and estrus) and treatment (5HT vs saline; 5HT + CAP vs CAP; M100907 vs Vehicle) as independent factors. 5HT content in interstitial fluid was reported as mean ± SEM ng/mL of 5HT across the stages of the estrous cycle and analyzed by ordinary two-way ANOVA. CGRP release was reported as mean ± SEM percent baseline levels and were analyzed by unpaired t-test or two-way ANOVA. The Grubb's test (GraphPad Quick Calcs Online, the extreme studentized deviate method; [(mean-value) / standard deviation]) was used to exclude a single outlier within an experimental group if present. Further, animals were removed from the study when environmental factors disrupted behavior testing (e.g. significant noise in the facility was noted

by the experimenter during testing). Bonferroni's correction was used to calculate *a priori* pairwise comparisons.

Results

Peripheral 5HT evokes significant orofacial nocifensive behaviors in female rats during proestrus and estrus, and in ovariectomized rats

We first tested whether peripheral injection of 5HT into the rat vibrissal pad evoked significant orofacial nocifensive behaviors across the estrous cycle in female rats compared to males and ovariectomized females. There was a significant three-way interaction of time by treatment by stage [$F(29.448, 116) = 1.612$; $p \leq 0.05$] and a significant main effect of time [$F(3.681, 116) = 14.558$; $p \leq 0.05$] and stage [$F(4, 116) = 2.816$; $p \leq 0.05$]. Across all animals tested (males and females combined), nocifensive behavior characteristically peaks in the initial 0–18 min post-5HT injection in a dose-dependent manner and wanes by 30 min post-5HT injection (see Figure 3.1A). In male rats, ovariectomized females, and diestrus females, no significant 5HT-evoked nocifensive behaviors were observed and forelimb swipes were comparable to saline injected controls (see Figure 3.1B-D; $p > 0.05$). 3 µg 5HT evoked significant nocifensive behaviors at 13–18 min in proestrus females (see Figure 3.1E; $p \leq 0.05$) and at 7–12 min in ovariectomized females ($p \leq 0.05$), whereas estrus females only displayed significant pain behaviors during the initial 6 min post-5HT injection (see Figure 3.1F; $p \leq 0.01$). We did not observe any hindlimb swipes indicative of itch at the 5HT concentrations used in this study.

Peripheral 5HT enhances capsaicin-evoked orofacial nocifensive behaviors in male rats and in female rats during proestrus and estrus

We then tested whether the nociceptive behavior can be enhanced in the presence of 5HT by injecting 5HT with a low dose of capsaicin (CAP) and recording the behavior over a 30 min time period. There was a significant three-way interaction of time by treatment by stage

[$F(29.186, 108) = 1.717$; $p \leq 0.05$] and significant two-way interactions of time by treatment [$F(7.297, 108) = 2.483$; $p \leq 0.05$], time by stage [$F(14.593, 108) = 1.991$; $p \leq 0.05$], and treatment by stage [$F(8, 108) = 2.694$; $p \leq 0.05$]. Collectively (males and females combined), nocifensive behaviors were highest in the initial 0–18 min in the 3 µg 5HT + CAP group as compared to 1.5 µg 5HT + CAP or CAP (see Figure 3.2A). 3 µg 5HT + CAP evoked significant nocifensive behaviors in males at the 7–12 min as compared to the CAP group (see Figure 3.2B; $p \leq 0.001$). No significant differences were observed between any 5HT + CAP treatment group and CAP in OVX females and diestrus females (see Figure 3.2C, 3.2D; $p > 0.05$). In proestrus females (see Figure 3.2E), the lower dose of 1.5 µg 5HT + CAP evoked significant nocifensive behaviors at 7–12 min ($p \leq 0.05$), whereas, in estrus females 3 µg 5HT + CAP evoked significant nocifensive behaviors at 7-12 min (see Figure 3.2F) post-injection as compared to CAP ($p \leq 0.01$). When data points were collapsed and only CAP-evoked behaviors were compared across males, OVX, and cycling females, there was no significant difference between any groups ($p > 0.05$).

5HT-evoked orofacial nocifensive behaviors are blocked by antagonism of the excitatory $5HT_{2A}$ receptor subtype in female rats

Since the $5HT_{2A}$ receptor, an excitatory G_q protein-coupled subtype, is expressed in the trigeminal ganglia of male rats and is involved in potentiation of TRPV1 activity (Kaur et al., 2018; Loyd et al., 2011), we next determined whether the $5HT_{2A}$ receptor was involved in 5HT-evoked orofacial nocifensive behaviors in female rats, as indicated in males rats. As there was no significant effect of 5HT in males or females in diestrus, we only selected the proestrus and estrus females to receive the $5HT_{2A}$ antagonist. Further, because males only display 5HT-evoked nocifensive behaviors when capsaicin is present, capsaicin was added to the 5HT when treating the males with the $5HT_{2A}$ antagonist. Local pretreatment with the selective $5HT_{2A}$ receptor antagonist M100907 15 min prior to 3 µg 5HT injection significantly reduced the number of

forelimb swipes in female rats during proestrus and estrus at 13–18 min (see Figure 3.3A; $p \leq 0.05$). In male rats however, pretreatment with M100907 followed by a 3 μg 5HT + CAP injection did not attenuate orofacial nocifensive behaviors significantly (see Figure 3.3B; $p > 0.05$). The effect of $5HT_{2A}$ antagonism in females with capsaicin was not tested, as 5HT acts via 5HT GPCRs to sensitize TRPV1 and not via direct actions at TRPV1 (Salzer et al., 2019).

Cycling females have a significantly higher basal level of 5HT in interstitial fluid as compared to males

Next, we tested if local 5HT content in the vibrissal pad interstitial fluid following inflammation differed across males, ovariectomized females, and females in different phases of the estrous cycle by injecting CFA into the left vibrissal pad and saline in the right vibrissal pad. Twenty-four hours post-injection, cycling females in diestrus, proestrus, and estrus had significantly higher 5HT content in the interstitial fluid collected from saline treated vibrissal pad as compared to males ($p \leq 0.05$ for diestrus and $p \leq 0.01$ for proestrus/estrus). Since the 5HT content was comparable between proestrus and estrus females, the data from these two groups was collapsed in the graph. 5HT content post-CFA evoked inflammation was comparable across all groups (see Figure 3.4; $p > 0.05$).

E2 pretreatment significantly increases serotonergic potentiation of capsaicin-evoked CGRP release from trigeminal sensory neurons independent of the excitatory $5HT_{2A}$ receptor

Since excitatory 5HT receptor subtypes co-express with TRPV1 on male trigeminal sensory neurons leading to an enhanced CAP-evoked CGRP release (Loyd et al., 2011), we next determined if pretreatment with 5HT and E2 would further enhance this CGRP release in female trigeminal sensory neurons. Thus, we quantified the CGRP release from primary cultures of trigeminal sensory neurons pretreated with either E2 (50 nM), 5HT (100 μM), or a combination of E2 + 5HT before being stimulated with a low concentration of CAP (50 nM). When trigeminal

ganglia neurons were pretreated with only 5HT (see Figure 3.5A) or E2 (see Figure 3.5B), CGRP release was comparable to the vehicle group ($p > 0.05$). CAP evoked significant CGRP release indicative of TRPV1 activity (see Figure 3.5C; $F(1, 53) = p \leq 0.05$). Interestingly, a combination of 5HT + E2 pretreatment followed by CAP evoked significantly higher CGRP release as compared to the 5HT + vehicle and compared to pretreatment prior to CAP stimulation (see Figure 3.5D; $p \leq 0.05$). When trigeminal ganglia neurons were pretreated with the selective $5HT_{2A}$ antagonist, M100907, CGRP release was not attenuated and was comparable to the 5HT + E2 pretreated group (see Figure 3.6).

Discussion

The role of gonadal hormones and inflammatory mediators is implicated in numerous peripheral pain conditions. But, it is essential to understand whether cycling gonadal hormones play a role in orofacial pain behaviors and whether they can potentiate the neuromodulatory effects of 5HT on the trigeminal pain processing. Our lab has previously reported that during phases of the estrous cycle when hormones greatly fluctuate (proestrus and estrus), intraplantar 5HT injection into the hindpaw triggers significant thermal hyperalgesia and mechanical allodynia in female rats (Kaur et al., 2018). This concurs with a previous study in human tissues reporting that 5HT significantly enhances capsaicin-evoked CGRP release from human dental pulp extracted from females during the luteal phase of the menstrual cycle (Loyd, Sun, et al., 2012). Since the role of estrogen in trigeminal pain processing is controversial, with some studies reporting a pronociceptive role while others report an antinociceptive role (Averitt et al., 2019), we designed experiments to understand the role of estrogen fluctuations on serotonergic neuromodulation of trigeminal pain.

It is reported that E2 replacement therapy increases plasma 5HT levels (Blum et al., 1996) and can increase binding potential of excitatory 5HT receptors (Moses-Kolko et al., 2003).

In addition, E2 and 5HT have been linked in multiple trigeminal ganglia disorders like migraine and headache with E2 either increasing the levels of 5HT or expression of 5HT receptors (Paredes et al., 2019). In the present study, we report that (1) 5HT-evoked nocifensive pain behaviors are sexually dimorphic and dependent on hormone status, (2) 5HT potentiates capsaicin-evoked pain behaviors in males and in females during proestrus and estrus, (3) cycling females have significantly higher basal peripheral 5HT levels as compared to males, (4) estrogen enhances the serotonergic neuromodulation of capsaicin-evoked CGRP release in cultured trigeminal sensory neurons, and (5) blocking the $5HT_{2A}$ receptor attenuates nocifensive behavior in female rats but does not affect capsaicin-evoked CGRP release in cultured trigeminal sensory neurons.

Studying pain behaviors in intact cycling females and males in essential to understand if gonadal hormones play a role in pain processing (Becker et al., 2005). Here, we tested the ability of two different concentrations of serotonin to evoke spontaneous nocifensive pain behaviors in intact animals across both sexes and in ovariectomized females. Similar to previous reports of the transient, nociceptive effects of peripheral 5HT (Loyd et al., 2013), nocifensive behaviors in all tested animals (males and females combined) peaked within about 10–15 min post-5HT injection and returned to near baseline after 30 min. Interestingly, injection of 5HT into the hindpaw evokes significant pain in males (Kaur et al., 2018; Loyd et al., 2011), while similar physiological levels of 5HT injected at the vibrissal pad did not evoke pain behaviors in males until capsaicin was also present. This indicates that trigeminal sensory neurons may be more sensitive to the sexually dimorphic effects of 5HT, supporting the numerous and diverse craniofacial pain disorders that are often 3–4 times more common in women. 5HT is also major pruritogen (Akiyama & Carstens, 2013; Jinks & Carstens, 2002) and in the present study we did not observe itch behaviors at the low concentrations of 5HT used. It is likely that increasing 5HT to 5–10 μg

(Akiyama et al., 2010; Morita et al., 2015) would induce itch behaviors and it would be interesting to observe whether itch behaviors are also modulated by estrogen.

The greatest 5HT-evoked pain behaviors were observed during proestrus and estrus. In females, dynamic estrogen fluctuations during the estrous or menstrual cycle are associated with pain modulation (Kramer & Bellinger, 2009; Mogil, 2012) and our data contribute that fluctuating gonadal hormone levels (such as during proestrus to estrus) or a loss of gonadal hormones (such as with ovariectomy) contribute to heightened sensitivity to a pronociceptive mediator. Others have also reported increased pain behaviors during these phases of the estrous cycle. Notably, treatment with prolactin (Diogenes et al., 2006) and formalin (K.E, 2011) display greater pain behaviors in proestrus and estrus females. Nocifensive behaviors were comparable to the saline injected control group in males, ovariectomized females, and diestrus females. For males, testosterone has been widely documented to play a major role in antinociception and analgesia in animal and human models (Archey et al., 2019; Burris et al., 1991; Frye & Seliga, 2001). It remains possible that testosterone may be playing a protective effect on serotonergic pain processing in trigeminal sensory neurons. Interestingly, when 5HT and capsaicin were injected together, 5HT-evoked pain behaviors were then observed in males, as well as females in proestrus and estrus. In both males (only during the 7–12 min bout) and estrus females, 3 µg 5HT evoked significant capsaicin-evoked nocifensive behaviors, whereas, in proestrus females 1.5 µg 5HT evoked significant nocifensive behaviors. Note that neither the low dose of 5HT or capsaicin alone could not evoke significantly higher pain behaviors during proestrus, but when injected together pain behaviors emerged *and* were significantly higher compared to proestrus rats receiving 3 µg 5HT. This suggests a sensitizing role of 5HT on TRPV1, causing receptor activation at a lower threshold followed by desensitization at the higher dosage. We propose that the peaking level of E2 is contributing to sensitizing the serotonergic pain mechanism during

proestrus. As 5HT, via its excitatory GPCRs, can sensitize TRPV1, it is possible that a lower amount of peripheral 5HT can sensitize trigeminal nociceptors, which are then desensitized at the higher (doubled) amount of 5HT. Whereas, once E2 levels fall, these is a loss of sensitization of this mechanism and a higher amount of 5HT is required to elicit a pain response, like in males. Based on these findings, our future studies are focused on determining the complex mechanistic relationship between 5HT receptors and TRPV1 during estrogen receptor activity.

5HT in the periphery is majorly released by platelets, immune cells, and the enterochromaffin cells in the gut (Ni et al., 2008; Spohn & Mawe, 2017). The immune cells release 5HT in response to inflammation, where it acts as a pronociceptive and proinflammatory mediator. The 5HT content released at the inflammation site can be highly variable. Here, we report sexual dimorphism only in the basal levels of 5HT in orofacial interstitial fluid. Post-CFA injection, the 5HT levels are elevated across all groups, although all three groups of females trended higher than the male group. This can be explained by the fact that inflammation triggers 5HT release. Even though the levels of 5HT released post-inflammation are not significantly different, perhaps as there is a ceiling effect occurring, the presence of hormones and the action of 5HT on its receptors can underlie the dimorphic effects of 5HT in males and females. For instance, it has been shown that 5HT content in the masseter muscle is higher in fibromyalgia patients as compared to healthy individuals, causing increased pain and allodynia (Ernberg, Hedenberg-Magnusson, Alstergren, & Kopp, 1999; Ernberg, Hedenberg-Magnusson, Alstergren, Lundeberg, et al., 1999). Also, estrus cycle dependent hormonal fluctuations are reported to affect mast cell numbers, maturation and degranulation, which would explain the increased basal level 5HT in our study (Zierau et al., 2012).

A major excitatory receptor of 5HT in the periphery, $5HT_{2A}$, has been implicated in several pain conditions including irritable bowel syndrome, fibromyalgia, headache and migraine,

and temporomandibular joint disorder (TMD) (Sugiuar et al., 2004). This excitatory receptor is coupled to the $G_{\alpha q}$ subunit of the G-protein coupled receptor (GPCR) family activating phospholipase C and protein kinase C (Hoyer et al., 2002). The activated kinase can phosphorylate TRPV1 leading to sensitization of the receptor and exacerbation of pain, which provides an opportunity for this particular 5HT receptor to sensitize TRPV1. It has been shown that $5HT_{2A}$ is involved in thermal allodynia and mechanical hypersensitivity associated with neuropathy as treatment with a $5HT_{2A}$ antagonist attenuated pain behaviors (Thibault et al., 2008). Here, we report significant attenuation of 5HT-evoked nocifensive behaviors when females in proestrus or estrus were pre-treated with a selective $5HT_{2A}$ antagonist, M100907, prior to 5HT injection. This study was limited to examining the role of the $5HT_{2A}$ receptor, however several other 5HT receptors may also be involved and in a sexually dimorphic manner. We recently reported that the $5HT_{3A}$ is expressed in the TRPV1 population of female trigeminal sensory neurons across the estrous cycle, but does not play a major role in sex differences in 5HT-evoked pain behaviors (Kaur et al., 2021). In contrast, recent studies have reported a major role of the $5HT_3$ receptor in mediating serotonergic pain and itch in TG and DRG neurons (Domocos et al., 2020; Kilinc et al., 2017). Domocos et al., 2020 also found that $5HT_{1A}$ receptor modulates 5HT-evoked scratching in Wistar rats. Additionally, several other excitatory 5HT receptors have recently been reported to play a role in serotonergic pain and itch, including the $5HT_4$ and $5HT_7$ (Lopez et al., 2021; Morita et al., 2015; Ohta et al., 2006). Literature is limited on role of $5HT_{5A/B}$ and $5HT_6$ family in the TG system, but $5HT_{5A/B}$ mRNA expression has been reported in the TG (Manteniotis et al., 2013). While our current investigations are focused on which estrogen receptors are involved in exacerbating serotonergic pain, future studies are warranted to examine the role of $5HT_4$ and $5HT_7$ in sex differences in serotonergic pain.

The attenuation was not seen in male rats though there was a trend towards reduced pain behaviors in M100907 injected animals. It is of importance to note that in See Figure 3.2B, 5HT+capsaicin evoked pain behaviors only during the 7–12 min bout (average of 27.6 swipes), however in the follow-up experiment testing the role of $5HT_{2A}$ in See Figure 3B we observed that the effect was spread out between the 7–12 min bout (average of 11.75 swipes) and the 13–18 min bout (average of 16.25 swipes) indicating variability in the peak of 5HT-evoked pain behaviors in males. Thus, the $5HT_{2A}$ antagonist may be effectively reducing pain behaviors in males, but is not being detected as significant in our data as presented across time. Interestingly, in humans a specific single nucleotide variation in the $5HT_{2A}$ receptor, rs6313, has been shown to be associated with pinprick hyperalgesia, indicating an important role of the receptor in pain sensitization (Sachau et al., 2021). In support, the T/T genotype of the T102C polymorphism in $5HT_{2A}$ receptor has been associated with low pain thresholds in fibromyalgia patients (Gürsoy et al., 2001).

Several studies have also looked at the activation profile of TRPV1 receptor in presence of estrogen and inflammatory mediators. Rowan et al. demonstrated that after a short-term exposure to E2, bradykinin signaling is enhanced in trigeminal ganglia primary cultures (Rowan et al., 2010). In addition, 30 min E2 treatment has been reported to induce CGRP release in a dose dependent manner in cultured DRG cell lines (Pota et al., 2017). Similarly, we see an enhanced capsaicin-evoked CGRP release when TG primary cultures from OVX females are pretreated with both 5HT and E2. Individually, neither 5HT nor E2 potentiate the capsaicin-evoked CGRP release. This is particularly interesting given that 5HT can increase capsaicin-evoked CGRP and calcium signaling in male TG neurons (Loyd et al., 2011) and that excitability of TMJ neurons is highest in presence of E2 and inflammation (Flake et al., 2005). In females at a similar concentration, 5HT was unable to evoked CGRP release unless E2 was present. Further, $5HT_{2A}$

antagonism did not attenuate the potentiated capsaicin-evoked CGRP release indicating estrogen receptor activity might be necessary in this modulation. It is possible that the effect of E2 occurs via a non-genomic effect of a membrane-bound estrogen receptor given the rapid effects of E2 on 5HT potentiated CGRP release. However, we did not see an effect of a 15 min E2 treatment alone on CGRP release. The 5HT-potentiated CGRP release occurs after E2 has been interacting in the system for 30–45 minutes, given our experimental design of a 15 min pretreatment with E2, followed by a 15 min treatment with E2+5HT, followed by a 15 min treatment with E2+5HT+CAP. Thus, it is also possible that a nuclear estrogen receptor is involved via a rapid signaling mechanism (Chen et al., 2021; Marino et al., 2006). We are currently working to identify which nuclear and/or membrane estrogen receptors (ERα, ERβ, or GPER) are involved in potentiating the effects of 5HT on trigeminal sensory neurons. Previous studies have reported that ERα, ERβ, and GPER are present in trigeminal sensory neurons (Chen et al., 2021; Warfvinge et al., 2020). Given the controversial nature of E2 on pain and 5HT, it is likely that different estrogen receptors may modulate 5HT-evoked trigeminal pain in a disparate manner.

Overall, we report that the proinflammatory and pronociceptive mediator 5HT at the trigeminal sensory neurons triggers orofacial pain behaviors in a sexually dimorphic and estrogen-dependent manner. This work is the first to report that estrogen potentiates 5HT-evoked orofacial pain in female rodents and female trigeminal sensory neurons. Understanding how hormones potentiate serotonergic neuromodulation will provide us a deeper understanding of the trigeminal pain mechanisms that underlie the disproportionate prevalence of craniofacial and orofacial pain disorders in women, especially those disorders involving 5HT, and thus aid in development of sex-based therapeutics.

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Figures

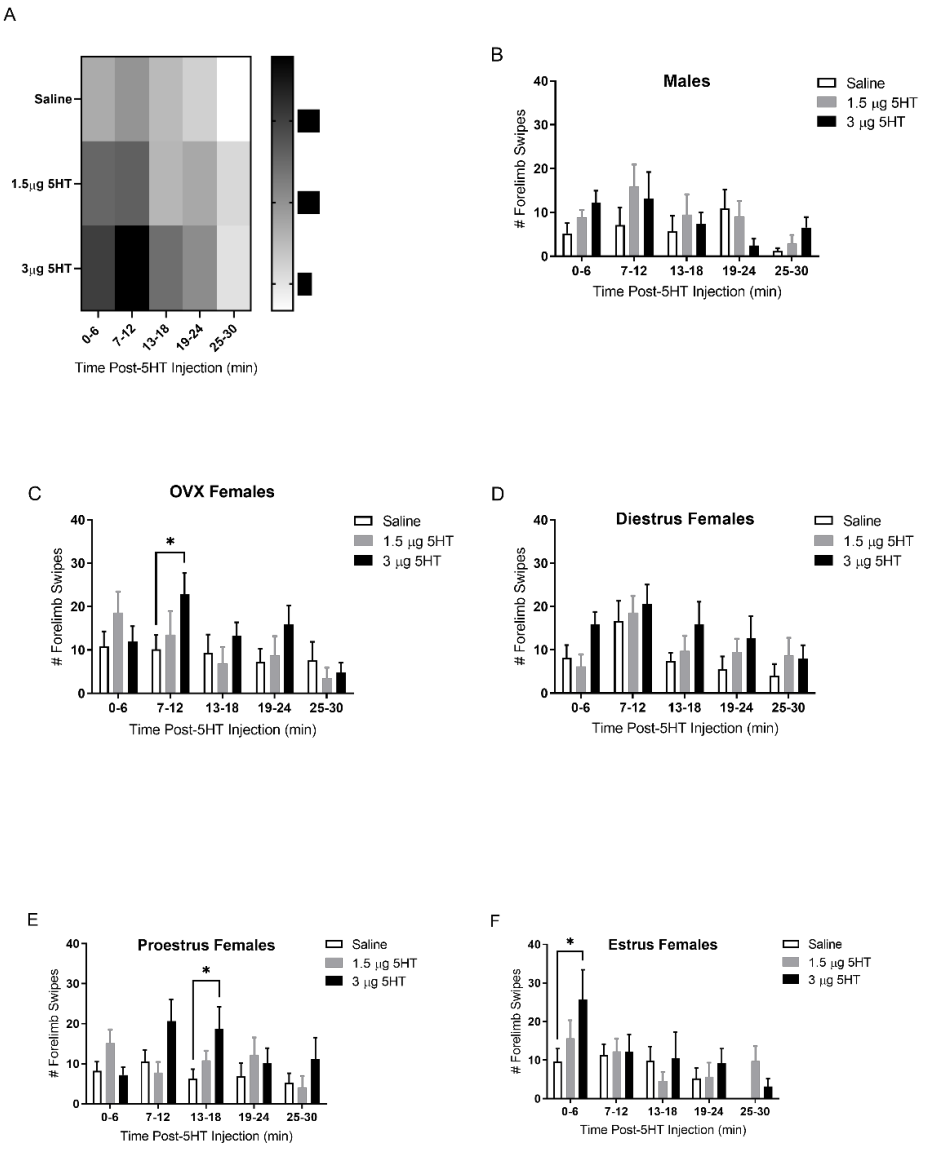

Figure 3.1. Peripheral 5HT evokes significant orofacial nocifensive behaviors in female rats during proestrus and estrus only. 5HT evoked pain behaviors peak in the initial 15 min in both male and female rats (data combined) as observed by increase in the heatmap grayscale gradient (A). In males (B) and diestrus females (D), pain behaviors evoked by 1.5 µg 5HT (grey bars) and 3 µg 5HT (closed bars) were not significantly different compared to saline vehicle control (open bars). 3 µg 5HT evokes significant nocifensive behaviors in ovariectomized (C), proestrus (E), and estrus (F) females as observed by an increase in the number of forelimb swipes. *Denotes a significant effect of 3 µg 5HT compared to saline at the respective time point with significance in pairwise comparisons tested at $p \leq 0.05$.

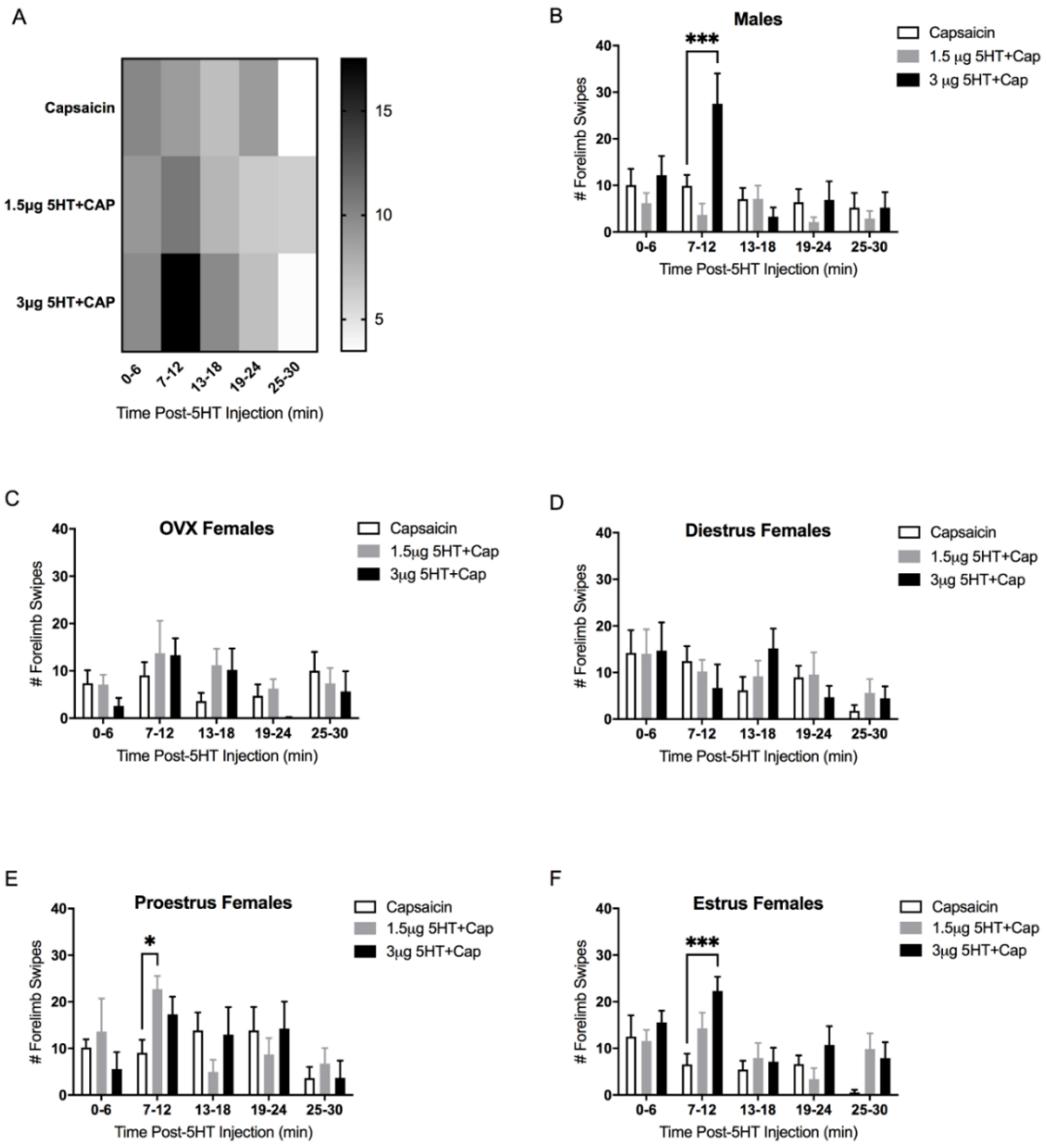

Figure 3.2. Peripheral 5HT enhances capsaicin-evoked orofacial nocifensive behaviors. 5HT-evoked pain behaviors peak in the initial 15 min in both male and female rats (data combined) as observed by increase in the heatmap grayscale gradient (A). 3 µg 5HT (closed bars) and not 1.5 µg 5HT (grey bars) evoked significant nocifensive behaviors in male rats (B) compared to capsaicin control (open bars) as observed by an increase in number of forelimb swipes. Neither doses of 5HT evoked significant pain behaviors in OVX females (C) and diestrus females (D). 1.5 µg 5HT evoked significant nocifensive behaviors in proestrus females (E) and 3 µg 5HT evoked significant nocifensive behaviors in estrus females (F). *Denotes a significant effect of 1.5 µg 5HT+capsaicin compared to capsaicin at the respective time point with significance in pairwise comparisons tested at $p \leq 0.05$. ***Denotes a significant effect of 3 µg 5HT+capsaicin compared to capsaicin at the respective time point with significance in pairwise comparisons reported at $p \leq 0.001$.

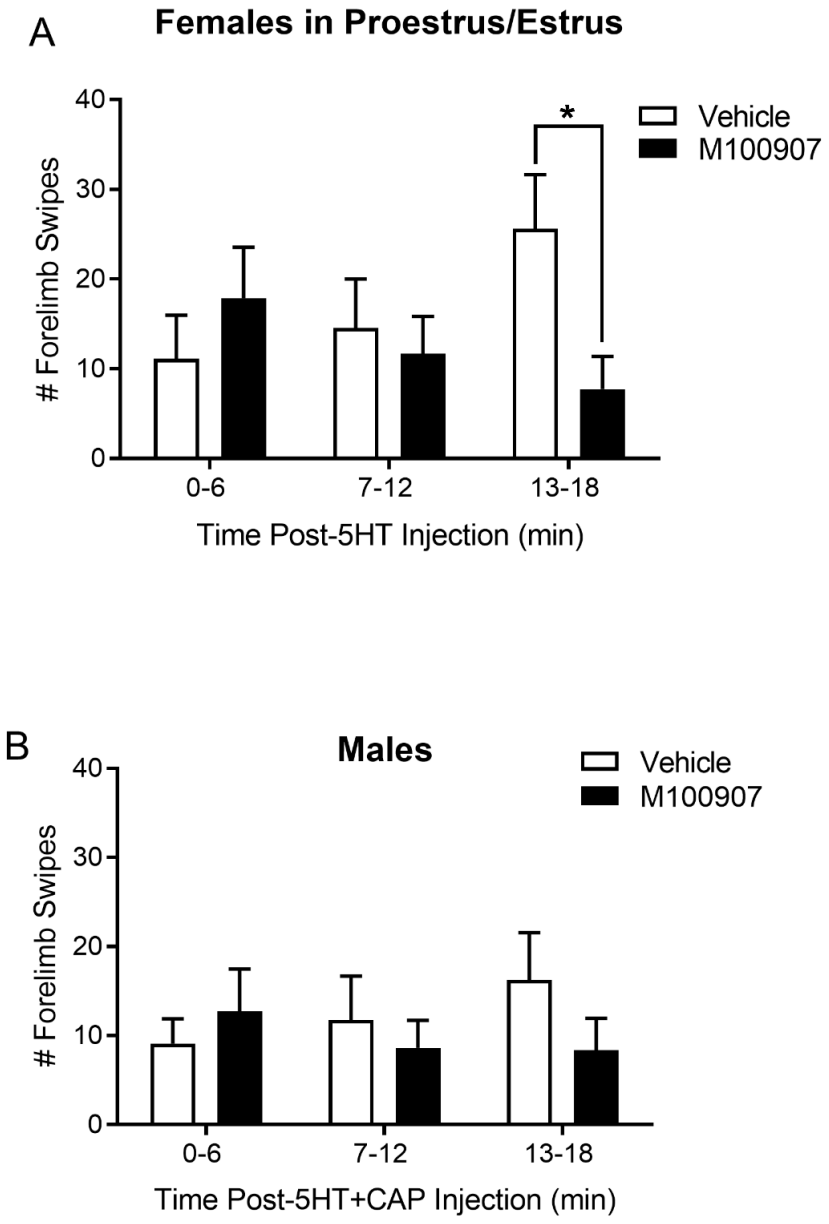

Figure 3.3. Blocking the excitatory $5HT_{2A}$ receptor attenuates 5HT-evoked orofacial nocifensive behaviors in female rats. M100907 (closed bars) pretreatment significantly reduced 5HT-evoked nocifensive behaviors in females during proestrus or estrus compared to the vehicle (open bars) pretreated group (A). M100907 pretreatment did not attenuate 5HT + capsaicin evoked nocifensive behaviors in male rats (B). *Denotes a significant effect of M100907 compared to vehicle at the respective time point with significance in pairwise comparisons tested at $p \leq 0.05$.

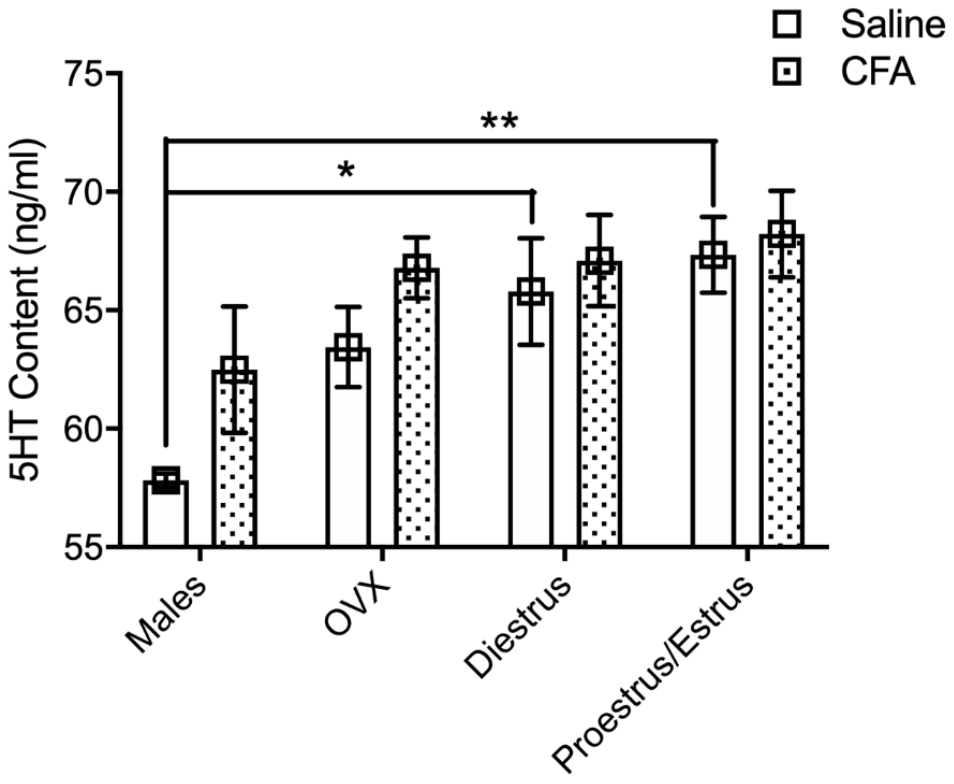

Figure 3.4. Basal levels of 5HT are elevated in the interstitial fluid from cycling females. Twenty-four hours after saline (open bars) injection, 5HT content was elevated in diestrus and proestrus/estrus females as compared to males. 5HT content was not significantly different in the CFA (dotted bars) injected groups. *Denotes a significant difference between 5HT content in pairwise comparisons tested at $p \leq 0.05$. **Denotes a significant difference between 5HT content in pairwise comparisons reported at $p \leq 0.01$.

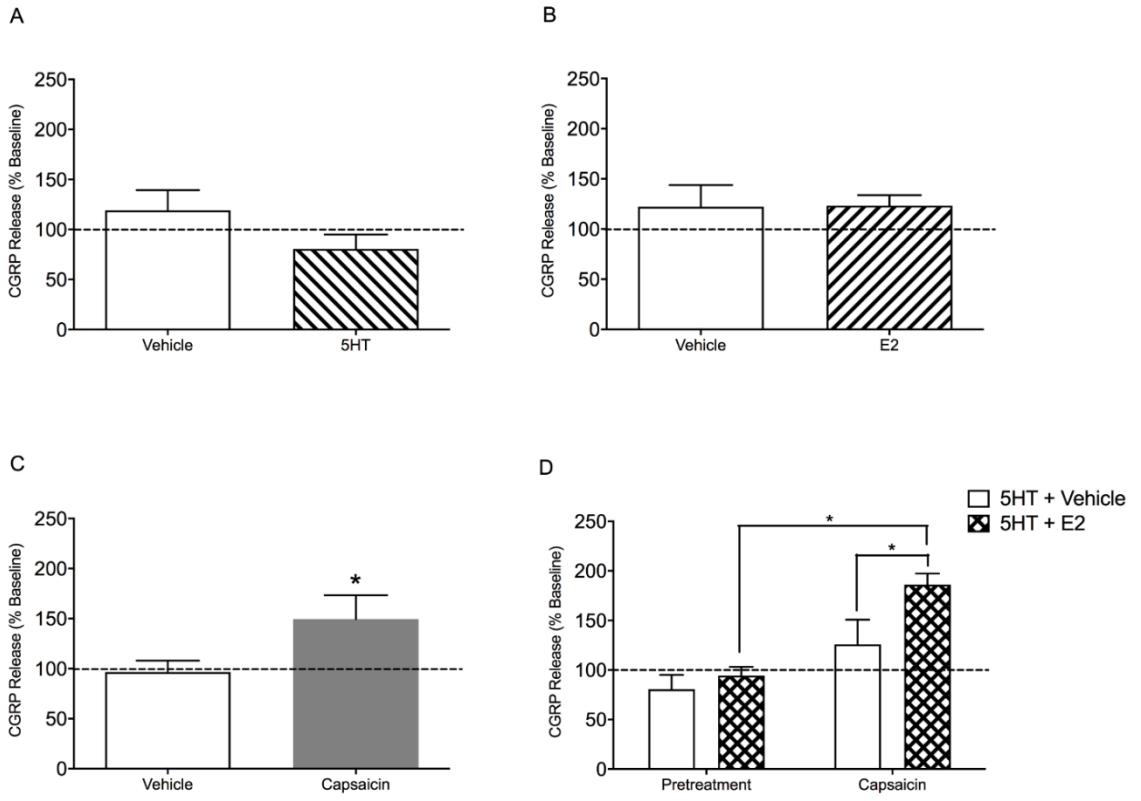

Figure 3.5. E2 pretreatment significantly increases serotonergic potentiation of capsaicin-evoked CGRP release. CGRP release from female rat primary trigeminal ganglia neuron cultures was comparable to vehicle (open bar) with 5HT only (right diagonals) pretreatment (A) and E2 only (left diagonals) pretreatment (B) as observed by percent change in baseline. Capsaicin (grey bar) treatment significantly increased CGRP release compared to vehicle as observed by percent change in baseline (C). E2+5HT (hatched bar) pretreatment significantly enhanced capsaicin-evoked CGRP release as compared to the vehicle and 5HT only treatment. *Denotes a significant increase in CGRP release compared to vehicle with significance in pairwise comparisons tested at $p \leq 0.05$.

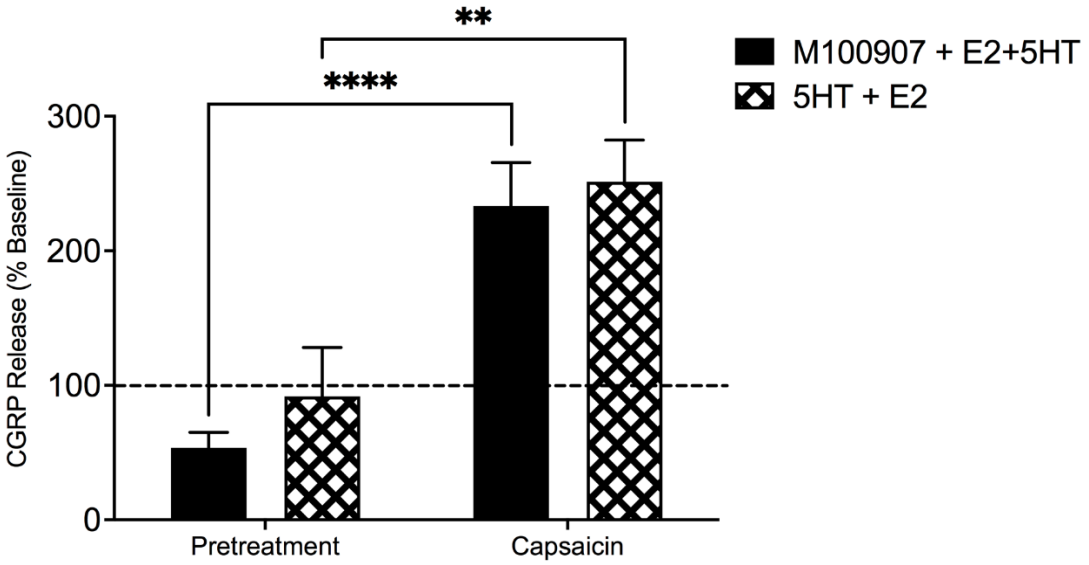

Figure 3.6. Antagonism of the $5HT_{2A}$ receptor does not attenuate CGRP release.
Pretreatment of female rat primary trigeminal ganglia neuron cultures with M100907 prior to treatment with E2 and 5HT (closed bars) did not attenuate capsaicin-evoked CGRP release compared to vehicle pretreatment (hatched bar). **Denotes significance compared to pretreatment with significance in pairwise comparisons tested at $p \leq 0.01$. ****Denotes significance compared to pretreatment in pairwise comparisons reported at $p \leq 0.0001$.

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CHAPTER IV

EXPRESSION OF SEROTONIN RECEPTOR SUBTYPE 3A ($5HT_{3A}$) ON RAT TRIGEMINAL SENSORY NEURONS

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Abstract

Serotonin (5-hydroxytryptamine, 5HT) is a neurotransmitter and proinflammatory mediator found largely in the peripheral nervous system where it can initiate pain signaling. 5HT binds a variety of 5HT receptors on sensory nerve endings specialized in detecting noxious stimuli, termed nociceptors. A subset of sensory neurons involved in pain signaling express the transient receptor potential vanilloid 1 ion channel (TRPV1), a pain generator. 5HT can both directly activate sensory neurons and sensitize TRPV1 leading to enhanced nociceptor sensitivity (peripheral sensitization). Previous studies in male rats reported that the 5HT receptor subtype 3A ($5HT_{3A}$) and TRPV1 are co-expressed on sensory neurons, but it is unknown if $5HT_{3A}$ and TRPV1 are co-expressed on female sensory neurons. Given that craniofacial pain disorders occur at a 2-3x greater prevalence in women, examining pain mechanisms in female trigeminal sensory neurons that innervate the craniofacial region is critical to advancing craniofacial pain management in women. Here we examined whether (1) 5HT acting via the $5HT_{3A}$ receptor produces sexually dimorphic orofacial pain behaviors in rats and (2) whether $5HT_{3A}$ receptor mRNA is expressed in trigeminal sensory neurons, including the TRPV1-expressing subpopulation, and increase pain signaling. We report that 5HT evokes pain behaviors in male and female rats, which was not significantly reduced by antagonism of $5HT_{3A}$. We performed *in situ* hybridization to label $5HT_{3A}$ and TRPV1 mRNA in trigeminal sensory neurons and found distinct cell populations with either $5HT_{3A}$ mRNA or TRPV1 mRNA in males and females; co-expression of $5HT_{3A}$ mRNA and TRPV1 mRNA was minimal. Further, $5HT_{3A}$ antagonism failed to reduce pain signaling in cultured trigeminal sensory neurons. These data suggest that the $5HT_{3A}$ subtype on trigeminal sensory neurons does not play a significant role in sexually dimorphic craniofacial pain disorders. Further research in the trigeminal sensory neurons should focus on other excitatory 5HT receptor subtypes. These data suggest that the $5HT_{3A}$ subtype on

trigeminal sensory neurons innervating the orofacial soft tissues does not play a significant role in sexually dimorphic craniofacial pain disorders. As previous studies have reported that granisetron reduces masseter muscle pain, $5HT_3$ may play a role in sex differences in myofascial pain disorders but not other craniofacial pain disorders.

Introduction

Trigeminal pain disorders, like migraine and temporomandibular joint disorder, disproportionately affect women with migraine being 2–3 times more prevalent in women (Berkley, 1997; LeResche et al., 2003). More than half of these women report menstrual-associated migraine in their reproductive years (Granella et al., 1993). Thus, gonadal hormone fluctuations [mainly estrogen (E2)] may be a possible explanation underlying these sex differences in trigeminal pain disorders. In addition, these disorders activate the immune system that release a milieu of pro-inflammatory mediators that can further modulate trigeminal pain signaling. One such proinflammatory and pronociceptive mediator released in the periphery is serotonin (5HT). 5HT is a neurotransmitter that is also a known peripheral algogen released by cells of the immune system and can act via a variety of excitatory ionotropic and metabotropic 5HT receptors to sensitize peripheral sensory neurons (meaning lower their activation threshold). Of the seven known 5HT receptors ($5HT_{1-7}$), $5HT_{3A}$ is an ionotropic receptor that belongs to the nicotinic acetylcholine superfamily of ion channels, whose activation results in the flow of sodium and potassium ions leading to an excitatory current in the sensory neuron (Maricq et al., 1991; Thompson & Lummis, 2007).

5HT is also known to sensitize a cation channel highly expressed in a subpopulation of trigeminal sensory neurons, the transient receptor potential vanilloid 1 (TRPV1) ion channel. TRPV1 is a thermosensor expressed in small- to medium-sized sensory neurons in the trigeminal ganglia that also acts as a pain generator (Loyd et al., 2013). Activation of TRPV1 by heat (>

42°C), capsaicin (the 'spicy' chemical in chili peppers), and protons results in an influx of calcium (Ca^{+2}) ions into the sensory neuron. The calcium influx results in the release of additional proinflammatory molecules, largely substance P and calcitonin gene related peptide (CGRP) (Kaur et al., 2018; Vay et al., 2012). 5HT can act through 5HT receptors to sensitize TRPV1 resulting in an increased Ca^{+2} influx and CGRP release (Loyd et al., 2013). We have previously reported that during periods of hormonal fluctuation, 5HT can evoke greater pain behaviors in female rats during the proestrus and estrus phases of the estrous cycle, characterized by rapid fluctuations in estrogen levels. During proestrus, estrogen rapidly rises and peaks and during estrus estrogen rapidly declines. Moreover, treatment with the $5HT_{2A}$ receptor antagonist, M100907, can attenuate these pain behaviors in both male and female rats suggesting an important role of excitatory 5HT receptors in pain processing (Kaur et al., 2018).

Of the known excitatory 5HT receptors, $5HT_{2A}$ and $5HT_{3A}$ have been shown to co-express with TRPV1 in male trigeminal sensory neurons, which provides an anatomical substrate for enhancing pain signaling in the neurons (Kaur et al., 2018). Studies have also reported that blocking the $5HT_{3A}$ receptor in the central nervous system reduces the sensitization of TRPV1, thus implicating an interaction between $5HT_{3A}$ and TRPV1 in pain signaling (Kim et al., 2014). Further, $5HT_{3A}$ antagonists are used as potent antiemetic drugs to block the release of 5HT in the gastrointestinal tract and reduce visceral pain (Theriot et al., 2020). Given that craniofacial pain disorders occur at a 2–3x greater prevalence in women, examining pain mechanisms in female trigeminal sensory neurons that innervate the craniofacial region is critical to advancing craniofacial pain management in women. Here we examined whether (1) 5HT acting via the $5HT_{3A}$ receptor produces sexually dimorphic orofacial pain behaviors in rats and (2) whether $5HT_{3A}$ receptor mRNA is expressed in trigeminal sensory neurons, including the TRPV1-expressing subpopulation, and increase pain signaling.

Materials and Methods

Subjects

A total of 16 adult male and 31 adult female Sprague–Dawley rats (200–300 g; Charles River Laboratories, Wilmington, MA) were used in the experiments. Rats were separated by sex and pair-housed in a 12:12-h light: dark cycle with ad libitum food and water access. All studies were approved by the Texas Woman's University Institutional Animal Care and Use Committee and conform to federal guidelines and guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain. This study was conducted in strict compliance with the Animal Welfare Act, implementing Animal Welfare Regulations, and the principles of the Guide for the Care and Use of Laboratory Animals.

Vaginal cytology

Vaginal lavages were performed between 0900AM to 1100AM at 24-h intervals beginning 2 weeks (at least two consecutive cycles; 10 days) before testing to confirm that all female rats were cycling normally. Daily records were maintained on the stages of their cycle through the day of experimental testing and tissue collection. Proestrus was identified as a predominance of nucleated epithelial cells and estrus was identified as a predominance of cornified epithelial cells. Diestrus 1 (or metestrus) was differentiated from diestrus 2 (or diestrus) by the presence of leukocytes (Becker et al., 2005; Loyd & Murphy, 2008; McLean et al., 2012).

Ovariectomy

A subset of female rats to be used for extraction of trigeminal ganglia (TG) for primary neuron cultures were deeply gas anesthetized (3% induction; 2.5% maintenance) by inhalation of Isothesia (isoflurane, USP, Henry Schein Animal Health, Dublin, OH) and a single incision was made across the abdomen. The abdominal muscle was opened and the ovary bundles were ligated with 4-O silk sutures, excised, and removed. The fascia was closed with 5-O silk suture and the

skin was closed with Vicryl sutures to prevent wicking. Rats were allowed 2 weeks for recovery and ovarian hormone dissipation. Trigeminal ganglia were removed from these animals following the 2-week recovery period using methodology described under the cell culture methodology section.

Behavior Testing

Square-shaped plexiglass boxes (30 x 30 x 30 cm) with mirrored sides were used to observe orofacial nocifensive behaviors. Rats were acclimated to the behavior testing apparatus 24 hours prior to testing. On the day of testing, rats were placed in the individual boxes immediately post-injection and nocifensive behavior was recorded with a video camera for a 30-min time period. The videos were manually quantified using iMovie software (Apple Inc., Mac OS) by counting the number of forelimb swipes over the injection site in 6 min bouts over an 18 min period and reported as a measure of spontaneous nocifensive behavior. Data was counted by an independent observer blind to the experimental condition.

Adult intact male and cycling female rats in either proestrus or estrus received an unilateral intradermal injection of the selective $5HT_{3A}$ antagonist granisetron (1 μM; 0.1 nmol / 100 μL; Sigma-Aldrich; Loyd, Chen, et al., 2012) or saline vehicle control (0.9%; 100 μL) into the vibrissal pad (n = 7–8 per sex and per treatment group). Fifteen-minutes after the pre-treatment, female rats received an injection of 5HT (3 μg / 50 μL) and the male rats received an injection of 5HT combined with capsaicin (3 μg 5HT + 1 μg CAP / 50 μL) at the same site. The male rats received the addition of capsaicin because our previous studies indicate 3 μg 5HT does not induce orofacial nocifensive behavior, while in females 3 μg 5HT produces significant orofacial nocifensive behavior. Immediately following the 5HT injection, orofacial nocifensive behavior were recorded and counted as described above.

In situ hybridization

Trigeminal ganglia were bilaterally extracted from males and cycling female rats two weeks following orofacial behavior testing and immediately frozen on dry ice. The tissues were stored at -80℃ until processing. The ganglia (n = 2/ animal) were embedded in Tissue-Tek Optimal Cutting Temperature (O.C.T) compound (Sakura Finetek USA) and cut into 30 µm sections on a Leica Cryostat CM3050 at -20℃. The slides were then stored at -80℃ until further processing. For *in situ* hybridization, the RNAscope Fluorescent Multiplex Assay was performed according to the manufacturer's specifications (ACD Biotechne) with optimizations performed for TG tissue. Briefly, slides were fixed in 4% paraformaldehyde, sequentially dehydrated in 50% ethanol, 70% ethanol, and 100% ethanol and stored at -20℃ overnight. The next day, slides were air-dried and incubated at 60℃ and a hydrophobic barrier was drawn using an ImmEdge Hydrophobic Barrier Pen (Vector Laboratories). Following a hydrogen peroxide and protease IV treatment, the slides were treated with either experimental probes (50:1 dilution; Table 1) or control probes and incubated at 40℃ for 2 hours. Slides were washed in buffer and amplification steps were performed with AMP-1, AMP-2, and AMP-3 (AMP indicates proprietary signal amplification molecules; ACD Biotechne), then slides were sequentially treated with OPAL fluorophores (see Table 4.1; Akoya Biosciences). Excess liquid was carefully drained from the slides and 1–2 drops of Prolong Gold antifade mounting medium (Fisher Scientific) with 4',6-diamidino-2-phenylindole (DAPI) were added and slides were cover slipped and air-dried overnight at room temperature. The slides were imaged using a Zeiss LSM 900 confocal microscope. Zeiss ZEN software was used to view and capture the images at 40X. The staining intensity was filtered by applying a laser power based on the control slides (Laser power - 570nm: 0.2%, 520nm: 1%) to remove low intensity pixels that represent nonspecific/background staining. All images were saved in tiff format.

Primary culture of trigeminal ganglia neurons

Trigeminal ganglia (n = 4 rats per 24-well plate run in triplicate) were extracted from adult ovariectomized female rats (~200 g) immediately following decapitation under brief gas anesthesia (3% isoflurane). Primary neuron cultures were prepared using previously described methods (Loyd et al., 2011). Briefly, trigeminal ganglia were suspended in Hank's balanced salt solution (HBSS) on ice and gently washed three times. After dissociation with collagenase (5%, Worthington Biochemical Corp, Lakewood, NJ) and trypsin (1%, Sigma–Aldrich) at 37 °C, the cells were suspended in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Waltham, MA) containing 10% fetal bovine serum, 1X glutamine, 1X penicillin-streptomycin, nerve growth factor (NGF, 100ng/ml; Harlan, Indianapolis, IN), and treated with mitotic inhibitors 5-fluoro-2'-deoxyuridine (3 μg/mL; Invitrogen) and uridine (7 μL/mL; Sigma-Aldrich). Cells were then lightly dissociated using a 20-gauge followed by a 23-gauge needle and then applied to 24-well poly-D-lysine-coated plates (Corning Inc., Corning, NY) and maintained in an incubator at 37 °C and 5% CO_2.

CGRP Release Assay

Primary cultures of trigeminal sensory neurons were grown and maintained for 5 days prior to running the CGRP release assay. The assay was performed using a protocol previously described (Loyd et al., 2011). Briefly, cultures were washed twice with 300 μL Hank's balanced salt solution (HBSS). Cells were then incubated in HBSS for 15 minutes and supernatant was collected for measurement of basal CGRP release from the neurons. The same cultures were then pretreated row-wise with either granisetron (100 nM) or 17β-estradiol (E2; 50 nM), followed by 5HT (100 μM) for 15 minutes and supernatant was collected for measurement of treatment-evoked CGRP release. Then cells in all the rows were stimulated with capsaicin (50 nM) to trigger CGRP release and supernatant was collected for measurement of the effects of treatment

on capsaicin-evoked CGRP release. CGRP in the superfusate was detected using a rat-specific CGRP ELISA (Cayman Chemical) and quantified using a Biotek ELx808 absorbance reader (Biotek). All experiments were conducted in duplicate with n = six wells per treatment group for a total of approximately 12 wells per group.

Data analysis

All data were analyzed and graphed with GraphPad Prism software version 9.0.0 (GraphPad, San Diego, CA). Orofacial nocifensive behavior and CGRP release data were expressed as mean ± standard error of the mean (SEM) and were analyzed by two-way ANOVA. Grubb's test was used to exclude a single outlier within an experimental group if present and Bonferroni's correction was used to calculate *a priori* pairwise comparisons.

Results

Granisetron does not significantly attenuate 5HT-evoked orofacial nocifensive behaviors in male or female rats

Since $5HT_{3A}$ is expressed in the trigeminal ganglia and is an excitatory ion channel, we first determined whether 5HT evokes nocifensive behaviors via the $5HT_{3A}$ receptor in male and female rats. 5HT alone evoked significant orofacial pain behaviors (saline pre-treatment group) at 7–12 minutes time bout after 5HT injection in female rats ($p \leq 0.05$). Local pre-treatment with the selective $5HT_{3A}$ receptor antagonist, granisteron, 15 minutes prior to either 5HT injection in females or 5HT+CAP injection in males did not significantly attenuate orofacial nocifensive behaviors (see Figure 4.1A and 4.1B; $p > 0.05$).

$5HT_{3A}$ receptor mRNA and TRPV1 ion channel mRNA are co-expressed in male and female trigeminal sensory neurons

We then performed *in situ* hybridization to determine whether $5HT_{3A}$ receptor mRNA co-expresses with TRPV1 ion channel mRNA in the trigeminal sensory neurons of the trigeminal

ganglia of male and female rats. We report $5HT_{3A}$ mRNA expression in distinct small and medium diameter neurons in the trigeminal sensory neurons of the trigeminal ganglia of males and cycling female rats (see Figure 4.2; magenta fluorescent punctate). TRPV1 ion channel mRNA was also observed in small and medium diameter neurons in the trigeminal sensory neurons of the trigeminal ganglia of males and cycling female rats (green fluorescent punctate). In both males and females, and across the estrous cycle, there was a subset of the sensory neuron population that co-expressed $5HT_{3A}$ receptor mRNA and TRPV1 ion channel mRNA (see Figure 4.2; white fluorescent punctate).

Granisetron does not significantly attenuate proinflammatory peptidergic activity in cultured trigeminal sensory neurons

As the proinflammatory peptide CGRP is released from sensory neurons when TRPV1 is activated, quantification of CGRP is a measure of nociceptive peptidergic activity in sensory neurons. Here we tested whether blocking the $5HT_{3A}$ receptor attenuates 5HT- and/or capsaicin-evoked CGRP release from cultured trigeminal sensory neurons extracted from ovariectomized female rats. When the neurons were treated with 5HT, E2, or granisetron alone (pretreatment) there was no effect on CGRP release (see Figure 4.3; $p > 0.05$). Capsaicin evoked significant CGRP release that was enhanced by 5HT and E2 (see Figure 4.3 grey bars; $p < 0.05$). CGRP release was not attenuated by pretreatment with the $5HT_{3A}$ antagonist granisetron ($p > 0.05$).

Discussion

Trigeminal pain disorders are more prevalent and severe in duration and intensity in women than men. Understanding the role of excitatory 5HT receptors in peripheral pain processing is important to the development of more effective, and even sex-specific therapeutics. We have previously reported that 5HT-evoked pain behaviors are increased during phases of the rodent estrous cycle when gonadal hormones are greatly fluctuating and that blocking the Gq-

coupled $5HT_{2A}$ receptor attenuates E2-enhanced pain behaviors (Kaur et al., 2018). In this study we report that (1) blocking the $5HT_{3A}$ receptor does not attenuate 5HT-evoked orofacial nocifensive pain behaviors, (2) $5HT_{3A}$ mRNA co-expresses with TRPV1 on male and female trigeminal sensory neurons, and (3) blocking the $5HT_{3A}$ receptor does not attenuate capsaicin-evoked CGRP release from cultured trigeminal sensory neurons.

Previous studies have reported that blocking the excitatory $5HT_{2A}$ and $5HT_{3A}$ receptors attenuates pain signaling in male cultured trigeminal sensory neurons (Loyd, Chen, et al., 2012; Loyd et al., 2013; Loyd et al., 2011). In support, $5HT_2$ and $5HT_4$ receptor antagonists attenuate 5HT-potentiated current in mouse DRG neurons (Sugiura et al., 2004). In the present study, we report that orofacial pain behaviors were not attenuated in presence of granisetron. These data indicate that the ionotropic $5HT_3$ receptor is not necessary for 5HT-evoked pain in the vibrissal pad. It is likely that the $5HT_3$ receptor is involved in generating pain in male and female rats, but antagonism of only the $5HT_3$ receptor is not sufficient to reduce orofacial pain in male or female rats. Rather, the involvement of the other excitatory G-protein coupled 5HT receptors are key to orofacial pain, such as the $5HT_{2A}$ receptor. Interestingly, $5HT_3$ receptors are upregulated in the masseter muscle of women with myofascial pain compared to healthy controls indicating that $5HT_3$ receptors may be more involved in pain processing in pathological trigeminal nociceptors innervating painful muscle rather than other trigeminal nociceptors (Christidis et al., 2014). In support, granisetron significantly reduces muscle pain, but not thermal pain, in men and women (Ernberg et al., 2020; Louca et al., 2013).

Studies have also reported that $5HT_{1A,}$ $5HT_{1D,}$ $5HT_{2A}$ and $5HT_{3A}$ receptors co-localize with TRPV1 on male trigeminal sensory neurons (Loyd et al., 2011). To date, no studies had examined whether 5HT receptors are co-expressed with the TRVPV1 ion channel in female trigeminal sensory neurons. In the present study, we report that $5HT_{3A}$ mRNA co-expresses with TRPV1 ion

channels on male and female trigeminal sensory neurons. The soma of nociceptors housed in the trigeminal ganglia are typically small- to medium-sized in diameter (~15–35 μm). $5HT_{3A}$ receptor mRNA and TRPV1 ion channel mRNA were expressed in the small- to medium-sized sensory neuron population. As the TRPV1 ion channel in a major pain generator, the population of sensory neurons that express TRPV1 are classified as nociceptors, though not all nociceptors express TRPV1 ion channels. Thus, our data provide evidence that $5HT_{3A}$ receptors are expressed by a subpopulation of nociceptors in the trigeminal ganglia. Regions with white fluorescent punctate indicating a cell expressing both TRPV1 mRNA and $5HT_{3A}$ receptor mRNA was observed in both sexes and across the four stages of estrous cycle. While $5HT_{3A}$ receptors appear to be localized on nociceptors in the trigeminal ganglia, our behavioral data indicate that blocking only the peripheral $5HT_{3A}$ receptors is not sufficient to reduce orofacial pain. We are currently examining whether $5HT_{2A}$ receptor mRNA is co-expressed in the TRPV1 population of trigeminal nociceptors and whether blocking peripheral $5HT_{2A}$ receptors is sufficient to reduce orofacial pain in female rats.

CGRP release is a measure of nociceptive peptidergic activity in sensory neurons and studies have correlated high CGRP release to worsened migraine symptoms in women (Goadsby et al., 1990; Hansen et al., 2010). Moreover, studies have reported that 5HT enhances capsaicin-evoked CGRP release from rat sensory neurons (Loyd et al. 2011) and female human tooth pulp (Loyd, Sun, et al., 2012). In concurrence with the *in vivo* arm of this study, our *in vitro* examination of the effects of granisetron on capsaicin-evoked CGRP release found that blocking the $5HT_3$ receptor does not alter 5HT-evoked pain signaling.

Conclusion

Overall, the present study indicates that while the ionotropic $5HT_3$ receptor may be involved in 5HT-evoked orofacial pain in female rats, selective antagonism of the $5HT_3$ receptor

with granisetron is not sufficient to reduce 5HT-evoked orofacial pain and thus not involved in the sexually dimorphic effects of peripheral 5HT on orofacial pain. Thus, it is more likely that a metabotropic 5HT receptor needs to be blocked in order to reduce 5HT-evoked orofacial pain in female rats. Further, this study is the first to report that $5HT_{3A}$ receptor mRNA is co-expressed with TRPV1 ion channel mRNA on both male and female trigeminal sensory neurons. Our data leads us to speculate that drugs targeting the peripheral $5HT_3$ receptor may not be sufficient to reduce trigeminal pain conditions not of muscle origin, such as migraine, in both men and women. Our current studies are now focused on the metabotropic 5HT receptors known to be localized to the trigeminal ganglia to determine whether targeting a different excitatory 5HT receptor may reduce orofacial pain.

Probe	Target	Channel	Fluorophore	Image Color	Working Dilution
Htr-3a	Serotonin receptor subtype 3A ($5HT_{3A}$) mRNA	C1	OPAL 570	Magenta punctate	1:1500
Trpv1	Transient Receptor Potential Vanilloid 1 ion channel (TRPV1) mRNA	C2	OPAL 520	Green punctate	1:1000

Table 4.1: Specifications of the RNAscope™ Fluorescent Multiplex Assay for *in situ* hybridization.

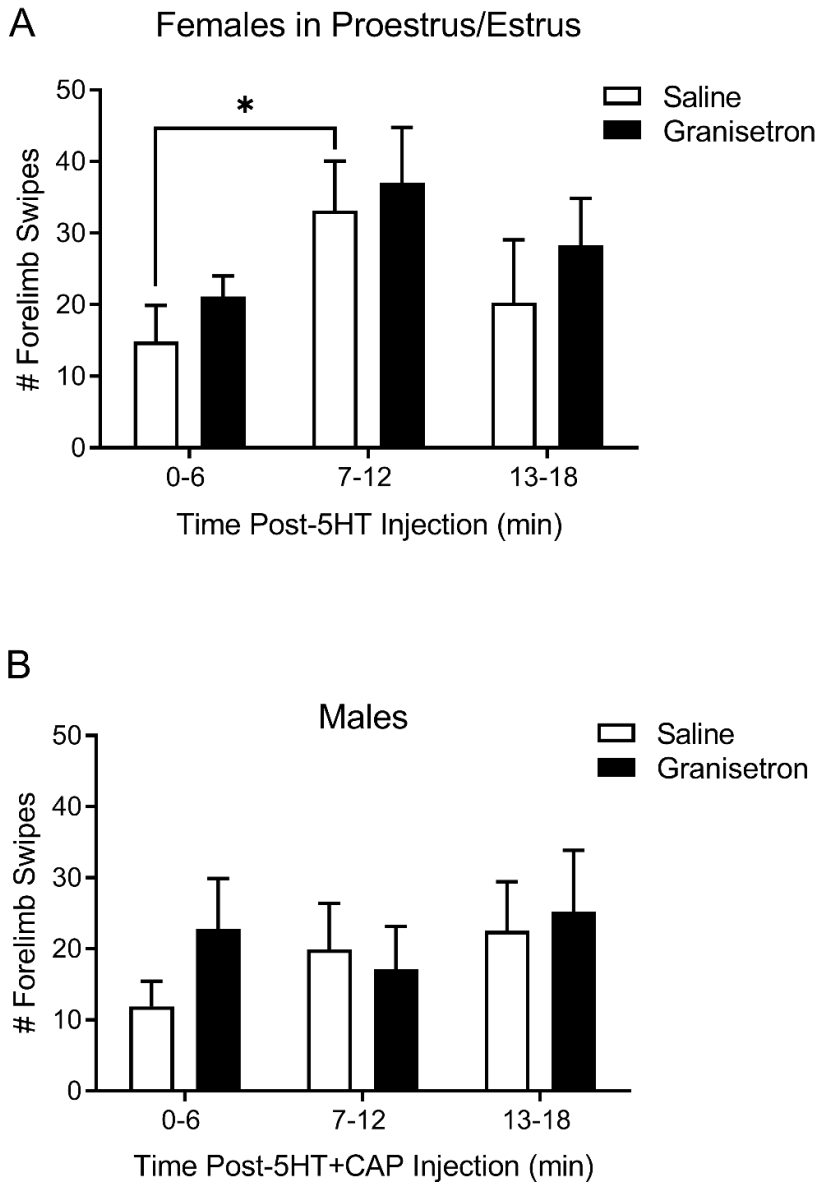

Figure 4.1: **Granisetron does not attenuate 5HT-evoked orofacial nocifensive behaviors in female and male rats.** Bar graphs illustrate the effects of saline pre-treatment (open bars) and granisetron pre-treatment (closed bars) on serotonin (5HT) alone in female rats (A) or 5HT with capsaicin (5HT+CAP) in male rats (B) on orofacial nocifensive behaviors recorded as the number of forelimb swipes from 0–18 minutes following injection into the vibrissal (cheek) pad. Asterisks indicate significant effect of 5HT on pain behaviors compared to vehicle treatment ($p < 0.05$). There was no significant effect of granisetron on pain behaviors ($p > 0.05$).

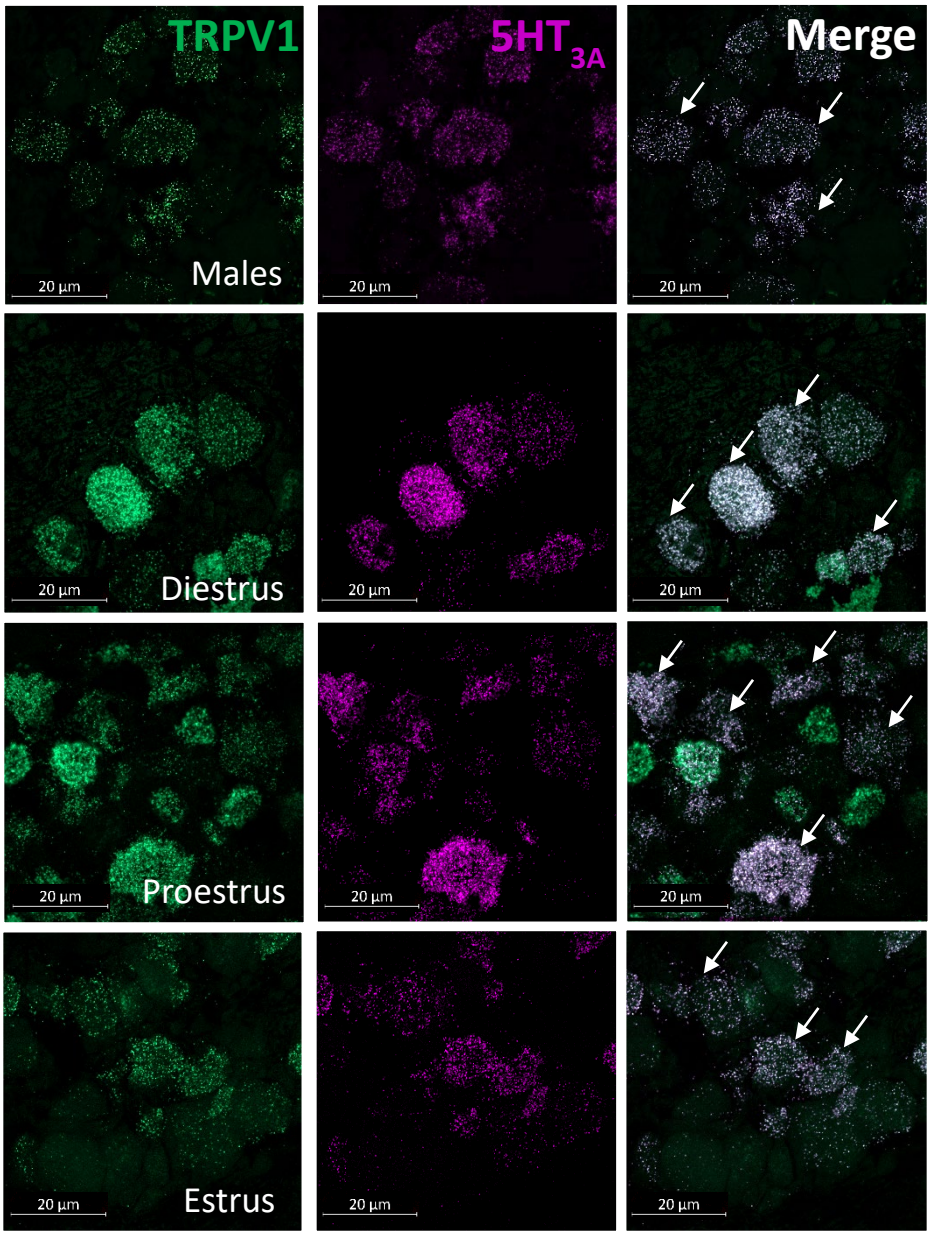

Figure 4.2: **$5HT_{3A}$ receptor mRNA and TRPV1 ion channel mRNA are co-expressed in male and female trigeminal sensory neurons.** Representative images of the expression of $5HT_{3A}$ receptor mRNA (magenta) and TRPV1 ion channel mRNA (green) in the trigeminal ganglia of male and cycling female rats across the three stages of the estrous cycle [diestrus, proestrus (P), and estrus (E)]. The first column illustrates TRPV1 ion channel mRNA expression, the second column illustrates $5HT_{3A}$ receptor mRNA expression, and the third column is an overlay of $5HT_{3A}$ receptor mRNA and TRPV1 ion channel mRNA. Arrows indicate sensory neurons co-expressing $5HT_{3A}$ receptor mRNA and TRPV1 ion channel mRNA (white).

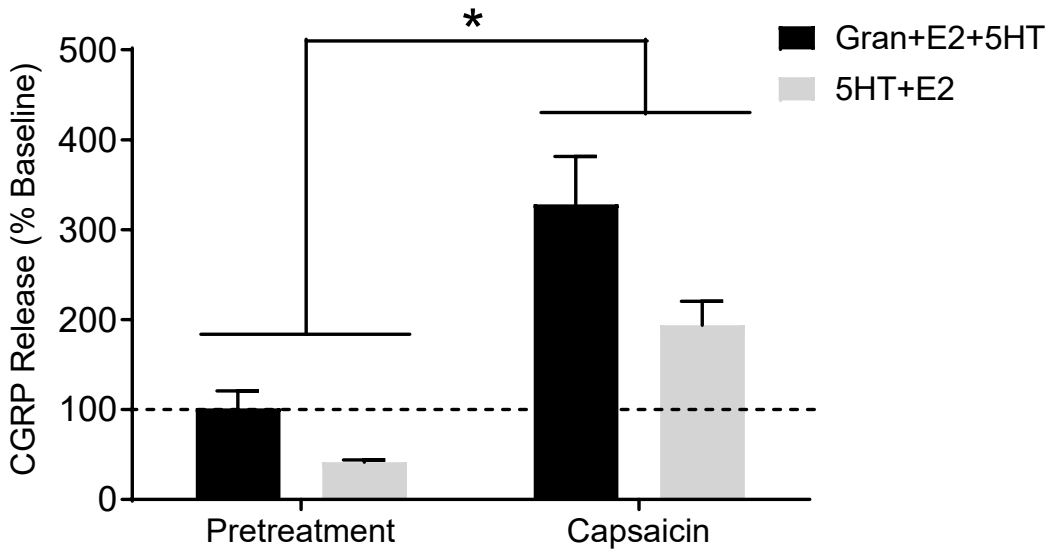

Figure 4.3: **Granisetron does not attenuate capsaicin-evoked CGRP release in trigeminal sensory neuron cultures.** Primary cultures pretreated with either the selective $5HT_3$ receptor antagonist granisetron (black pretreatment bar) or vehicle (grey pretreatment bar) prior to treatment with serotonin (5HT) and 17β-estradiol (E2) and stimulation with the TRPV1 agonist capsaicin. The asterisks indicate significance between pre-treatment vs capsaicin treatment. Asterisks indicate significant effect of 5HT+E2 on CGRP release as compared to vehicle ($p < 0.05$). There was no significant effect of granisetron on the enhanced CGRP release ($p > 0.05$).

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CHAPTER V

ESTROGEN MODULATION OF THE PRONOCICEPTIVE EFFECTS OF SEROTONIN ON FEMALE RAT TRIGEMINAL SENSORY NEURONS IS TIMING- AND DOSAGE-DEPENDENT AND REQUIRES ESTROGEN RECEPTOR ALPHA

A Paper to be Submitted for Publication in the

PAIN Journal

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Abstract

The role of the major estrogen estradiol (E2) on orofacial pain conditions remains controversial with studies reporting both a pronociceptive and antinociceptive role of E2. E2 modulation of peripheral serotonergic activity may be one mechanism underlying the female prevalence of orofacial pain disorders. We recently reported that female rats in proestrus and estrus exhibit greater serotonin (5HT)-evoked orofacial nocifensive behaviors compared to diestrus and males. Further coexpression of $5HT_{2A}$ receptor mRNA in nociceptive trigeminal sensory neurons that express transient receptor potential vanilloid 1 ion channels (TRPV1) contributes to pain sensitization. E2 may exacerbate orofacial pain via 5HT-sensitive trigeminal nociceptors, but whether low or high E2 contributes to orofacial pain and by what mechanism remains unclear. We hypothesized that steady-state exposure to a proestrus level of E2 exacerbates 5HT-evoked orofacial nocifensive behaviors in female rats, we explored the transcriptome of E2-treated females, and we determined which E2 receptor contributes to sensitization of female trigeminal sensory neurons. We report that a diestrus level of E2 is protective against 5HT-evoked orofacial pain behaviors, which increase with increasing E2 concentrations, and E2 differentially alters several pain genes in the trigeminal ganglia. Further, E2 receptors co-expressed with $5HT_{2A}$ and TRPV1 and enhanced capsaicin-evoked signaling in the trigeminal ganglia via ERα. Overall our data indicate that low, but not high, physiological levels of E2 protect against orofacial pain, and we provide evidence that ERα receptor activation, but not others, contribute to sensitization of nociceptive signaling in trigeminal sensory neurons.

Keywords: Estrogen, GPER, ERα, ERβ, Serotonin, Pain, $5HT_{2A}$ receptor, Trigeminal Sensory Neurons

Introduction

Numerous pain conditions that have similar prevalence in males and females during childhood are reported to be < 2X higher in females after menarche [10,22]. Pain conditions with a higher prevalence in women largely include disorders that occur in the trigeminal sensory system. Given the variability in trigeminal pain conditions over childhood, during menarche and the child-bearing years, and after the onset of menopause, the female gonadal hormones estrogen and progesterone clearly contribute to sexually dimorphic pain mechanisms. Progesterone and its metabolites play a neuroprotective role and have anti-inflammatory and antinociceptive properties [4,25,39,97]. Estrogen (E2) exerts both pro- and antinociceptive effects on trigeminal pain. The effects of E2 are multifactorial and may depend on gonadal hormone levels (steady-state vs fluctuating), dosage or concentration used, the type of pain or pain model being examined, and which estrogen receptor (ER) is bound (classical nuclear receptors ERα and ERβ vs the membrane bound G protein-coupled estrogen receptor GPER) [20,34,66].

One trigeminal pain mechanism that may be exacerbated by E2 is peripheral serotonergic modulation of trigeminal sensory neurons. Serotonin (5HT) has been implicated in several pain disorders more prevalent in women [29,32,36]. While 5HT in the central nervous system is often antinociceptive, 5HT is a robust pronociceptive mediator in the periphery activating rat [52,55,71,91,94] and human nociceptors [28,53,93]. 5HT triggers pain by acting on excitatory 5HT receptors, largely $5HT_{2A}$ and $5HT_3$, expressed on the transient receptor potential vanilloid 1 (TRPV1) population of trigeminal nociceptors [55,71,91]. Recently, we reported that 5HT evokes greater and longer-lasting pain behaviors in the female rat hindpaw [43] and the vibrissal pad [45] during proestrus (peaking E2) and estrus (declining E2), and likely involving the $5HT_{2A}$ receptor [43,45] rather than the $5HT_3$ receptor [44]. Further, we reported that E2 enhances the nociceptive effects of 5HT on the TRPV1 population of trigeminal sensory neurons [45]. This is supported by

our previous report that 5HT enhances release of calcitonin gene-related peptide (CGRP) from human dental pulp during the late luteal phase of the menstrual cycle, when E2 levels peak and rapidly decline [53].

While it is established that E2 modulates the serotonergic system and 5HT is pronociceptive at trigeminal nociceptors in a sexually dimorphic manner, whether low or high E2 contributes to orofacial pain and by what mechanism remains unclear. We hypothesized that steady-state exposure to a proestrus level of E2 exacerbates 5HT-evoked orofacial nocifensive behaviors in female rats, we explored the transcriptome of E2-treated females, and we determined which E2 receptor contributes to sensitization of female trigeminal sensory neurons. Here, we (1) manipulated E2 levels in adult female rats and measured 5HT-evoked nocifensive behavior, (2) examined the transcriptome of the trigeminal ganglia extracted from the hormone-manipulated female rats, (3) probed the TRPV1 population of female rat trigeminal sensory neurons for ERα, ERβ, GPER, and $5HT_{2A}$ receptor mRNA, and (4) pharmacologically targeted the estrogen receptors in primary cultures of trigeminal sensory neurons to determine which estrogen receptor(s) are involved in serotonergic potentiation of nociceptive signaling.

Methods

Subjects

A total of 3 adult male and 147 adult female Sprague–Dawley rats (200–300 g; Charles River Laboratories, Wilmington, MA) were used in the experiments. Rats were separated by sex and pair-housed in a 12:12-h light: dark cycle with ad libitum food and water access. All studies were approved by the Texas Woman's University Institutional Animal Care and Use Committee and conform to federal guidelines and guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain. This study was conducted in strict

compliance with the Animal Welfare Act, implementing Animal Welfare Regulations, and the principles of the Guide for the Care and Use of Laboratory Animals.

Vaginal cytology

Vaginal lavages were performed between 0900AM to 1100AM at 24-h intervals beginning 2 weeks (at least two consecutive cycles; 10 days) before testing to confirm that all female rats were cycling normally. Daily records were maintained on the stages of their cycle throughout experimental testing. Proestrus was identified as a predominance of nucleated epithelial cells and estrus was identified as a predominance of cornified epithelial cells. Diestrus 1 (or metestrus) was differentiated from diestrus 2 (or diestrus) by the presence of leukocytes [8,54,63]. When no significant differences were noted in behavior of diestrus 1 and diestrus 2 animals, these data were pooled and reported as such.

Ovariectomy

Female rats (n = 135) were deeply gas anesthetized (3% induction; 2.5% maintenance) by inhalation of Isothesia (isoflurane, USP, Henry Schein Animal Health, Dublin, OH) and a single incision was made across the abdomen. The abdominal muscle was opened and the ovary bundles were ligated with 4-O silk sutures, excised, and removed, as previously described [101]. The fascia was closed with 5-O silk suture and the skin was closed with Vicryl sutures to prevent wicking. Rats were allowed 2 weeks for recovery and ovarian hormone dissipation.

Drugs

β-estradiol 3-benzoate (E2; Sigma–Aldrich, St. Louis, MO) was dissolved in corn oil, stored as a 2 mg/mL solution at room temperature, and serial diluted to 2 µg/mL, 20 µg/mL, or 200 µg/mL on the day of use. Serotonin hydrochloride (5HT; Sigma–Aldrich) was dissolved in double-distilled water and diluted in 0.9% sterile saline or Hank's balanced salt solution (HBSS) buffer immediately prior to each use. Capsaicin (CAP; Sigma–Aldrich) was dissolved in 100%

ethanol in a fume hood and aliquots were stored at -20°C as 100 mM stocks. Capsaicin was freshly diluted in HBSS buffer prior to each use. β-Estradiol (E2; Sigma–Aldrich) was dissolved in 100% ethanol to create a 10 mM stock solution that was further diluted in HBSS buffer for a working solution of 50 nM. β-Estradiol 6-(O-carboxy-methyl) oxime: BSA (E2-BSA) was dissolved in HBSS to create a stock solution of 10 μM and was used at a final concetration of 50 nM. The estrogen receptor agonists/ antagonists, ICI 182,780, rel-1-[4-(6-bromo-1,3-benzodioxol-5-yl)-3aR,4S,5,9bS-tetrahydro-3H-cyclopenta[c]quinolin-8-yl]-ethanone (G1), 1,3,5-Tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT), 2,3-Bis(4-hydroxyphenyl)propionitrile (DPN), Methyl-piperidino-pyrazole hydrate (MPP), and 4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]-pyrimidin-3-yl]phenol (PHTPP) were dissolved in DMSO to prepare the stock solutions and further diluted in HBSS to final concentrations as listed in Table 5.1.

Silastic hormone capsule preparation and implantation

Capsules were prepared as previously described [61]. Briefly, Silastic ® tubing (0.058 in. ID x 0.077 in. OD (Dow Corning, Midland, Michigan) was cut into 1 cm capsules, sealed on one end with medical adhesive (Factor II, Inc., Lakeside, AZ), and allowed to dry overnight. E2 was weighed as 5%, 10%, or 20% of cholesterol and was mixed thoroughly to create a uniform powder. The hormone: cholesterol mixture was then packed into the capsule by tapping it gently. Once filled, the other end of the tubing was sealed and allowed to dry overnight. The capsules were then washed twice in 100% ethyl alcohol and immersed in 0.1M PBS for 48 hours. The capsules that sunk in 0.1M PBS were discarded and the floating capsules (indicating properly sealed ends) were used for implantation. The capsules were implanted at a final dose of 5% (physiological level comparable to diestrus; 15–20 pg/ml; one capsule), 20% (physiological level comparable to proestrus; 40–60 pg/ml; 2 capsules of 10%), 40% (supraphysiological level; 2

capsules of 20%) E2, or control (2 capsules of 100% cholesterol) [61]. The capsules were implanted subcutaneously into the back of ovariectomized female rats under anesthesia (2.5–3% isoflurane) using aseptic technique.

Orofacial nocifensive behavior testing

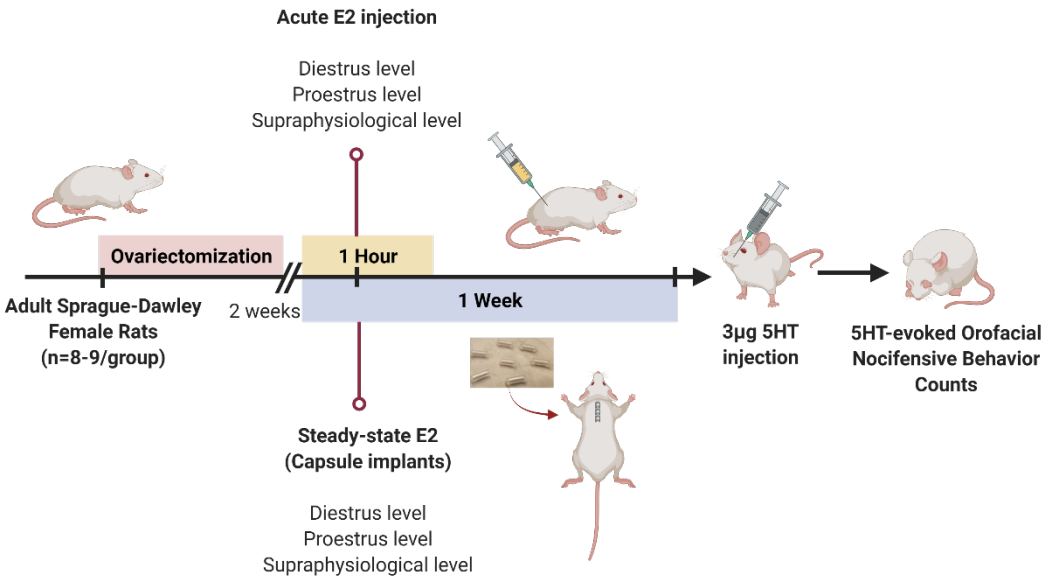
Orofacial nocifensive behaviors were quantified as previously described [45]. Briefly, rats were placed in individual mirrored boxes and nocifensive behavior was recorded with a video camera for a 30-min period. The videos were manually quantified using iMovie software (Apple Inc., Mac OS) by counting the number of forelimb swipes directed at the injection site in 6 min bouts over a 30 min period and reported as a measure of spontaneous nocifensive behavior. Rat forelimb swipes have been characterized in the literature as spontaneous nocifensive behavior distinct from grooming and itch behaviors [86,90]. Data was collected by two independent observers blind to the experimental condition and their values were averaged. If there were substantial differences between the two observer's counts (> 5 swipes), they were re-evaluated together to concur for final reporting.

Orofacial nocifensive behavior was tested in ovariectomized rats with E2 manipulation by either steady-state E2 treatment using silastic capsules (noted in above methodology section) or acute E2 injections (n = 8–9 per experimental treatment group). For the acute E2 group, ovariectomized females were gas anesthetized (2.5–3% isoflurane) and received a single subcutaneous injection (at the flank) of either 2 μg/mL E2 (physiological level comparable to diestrus; 15–20 pg/ml), 20 μg/mL E2 (physiological level comparable to proestrus; 40–60 pg/ml), 200 μg/mL E2 (supraphysiological level), or vehicle control (corn oil; 1 ml/kg per animal) [72]. One hour following acute E2 injections or 1 week following E2 capsule implantation, all rats received a single intradermal injection of 3 μg/50 μL 5HT into the right vibrissal pad (30-gauge needle). The number of forelimb swipes over the injected area were counted as described above

as 5HT-evoked nocifensive behaviors (see timeline below). Following completion of behavior testing, rats were rapidly decapitated and bilateral trigeminal ganglia were collected and stored at -80°C until further use.

Timeline 5.1

Illustration of time course of groups and treatments for behavior studies. Created with BioRender.com.



RNA isolation and RNA sequencing (RNA-seq)

RNA was extracted from the trigeminal ganglia collected from the hormone treated rats using the Qiagen RN-easy kit protocol. Briefly, right trigeminal ganglia (n = three per hormone treatment group as described above) was lysed using a Bead Mill Homogenizer (VWR, Radnor, PA). The lysate was passed through a gDNA spin column (Qiagen, Germantown, MD) to eliminate genomic DNA and flow-through was used for RNA isolation. A 1:1 70% molecular-grade ethanol was added to the flow-through and passed through a RNeasy spin column (Qiagen).

The RNA collected on the membrane of the spin column was washed three times with provided buffer and the column was transferred to a sterile, RNase-free tube. RNA was eluted by adding 30–50 µL RNase-free water to the column and centrifuging for 1.5 min at 10,000 rpm. RNA was quantified using a Nanodrop™ spectrophotometer (ThermoFisher Scientific, Waltham, MA). RNA quality was confirmed by A260/280 ratio and only the samples that had a ratio of ≥2 and a concentration > 20 ng/µL were sent for sequencing to Novogene (sequencing lab at The University of California at Davis campus). Novogene confirmed the RNA purity and integrity of the samples using a NanoPhotometer® spectrophotometer and the RNA Nano 6000 Assay Kit (Bioanalyzer 2100 system), respectively. Samples with RNA integrity number (RIN) > 8 were used for library preparation and sequencing on an Illumina platform. Genes with p value ≤0.05 were considered as differentially expressed genes (DEGs) and were compared against three databases specific to pain genes [Human Pain Genetics Database, Pain Research Forum - Pain Genes Database, and Database curated by Pokhilko et al.,2020 [77]]. Molecular function analysis were was performed using PANTHER database and heat maps of differentially expressed genes were created using fold changes provided by Novogene.

In situ hybridization

Trigeminal ganglia were bilaterally extracted from intact males and cycling female rats (*n* = 4 per sex and stage of estrous cycle) and immediately frozen on dry ice. The tissues were stored at -80°C until processing. Bilateral ganglia were embedded in Tissue-Tek Optimal Cutting Temperature (O.C.T) compound (Sakura Finetek, Torrence, CA) and sectioned at 30 µm on a Leica Cryostat CM3050 (Leica Biosystems, Buffalo Grove, IL) set at -20°C. The slides were then stored at -80°C until further processing. For *in situ* hybridization, the RNAscope® Fluorescent Multiplex Assay was performed according to the manufacturer's specifications (Advanced Cell Diagnostics bio-techne, Newark, CA) with optimizations performed for trigeminal ganglia tissue.

Briefly, slides were fixed in 4% paraformaldehyde, sequentially dehydrated in 50% ethanol, 70% ethanol, and 100% ethanol and stored at -20°C overnight. The next day, slides were air-dried and incubated at 60°C and a hydrophobic barrier was drawn using an ImmEdge Hydrophobic Barrier Pen (Vector Laboratories, Burlingame, CA). Following a hydrogen peroxide and protease IV treatment, the slides were treated with experimental probes against $5HT_{2A}$ and TRPV1 (or provided positive and negative control probes) and incubated at 40°C for 2 hours. Slides were washed in buffer and amplification steps were performed with kit-provided AMP-1, AMP-2, and AMP-3, then slides were sequentially treated with OPAL fluorophores (OPAL 690 and OPAL 520; Akoya Biosciences, Marlborough, MA). Excess liquid was carefully drained from the slides and 1–2 drops of Prolong Gold antifade mounting medium (Fisher Scientific) with 4',6-diamidino-2-phenylindole (DAPI) were added and slides were cover slipped and air-dried overnight at room temperature. The slides were imaged using a Zeiss LSM 900 confocal microscope. Zeiss ZEN software was used to view and capture the images at 40X. Every 1:4 sections across the V1/V2 area of the trigeminal ganglia were taken under the same gain and quantified using NIH ImageJ software.

Primary culture of rat trigeminal ganglia neurons

Trigeminal ganglia (n = 4 rats per 24-well plate run in triplicate) were extracted from adult OVX female rats (~200 g) immediately following decapitation. Primary neuron cultures were prepared using previously described methods [55,74]. Briefly, trigeminal ganglia were suspended in HBSS on ice and gently washed three times. After dissociation with collagenase (5%, Worthington Biochemical Corp, Lakewood, NJ) and trypsin (1%, Sigma–Aldrich) at 37 °C, the cells were suspended in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Waltham, MA) containing 10% fetal bovine serum, glutamine, penicillin-streptomycin, nerve growth factor (NGF, 100ng/ml; Harlan, Indianapolis, IN), and mitotic inhibitors (5-fluoro-2'-deoxyuridine and

uridine). Cells were then lightly dissociated using a 20-gauge followed by a 23-gauge needle and then applied to 24-well poly-D-lysine-coated plates (Corning Inc., Corning, NY) and maintained in an incubator at 37 °C and 5% CO_2.

CGRP release assay

Trigeminal ganglia primary cultures were maintained for 5 days prior to running the CGRP release assay. The assay was performed using a protocol previously described [55]. Table 5.1 provides details on the drugs used in this experiment. Briefly, cultures were twice incubated for 15 minutes each with 300 μL HBSS. Superfusate was collected from the second incubation for measurement of the basal level of CGRP release prior to drug treatments. Cells were then treated for 15 minutes in either estrogen receptor agonists, antagonists, or vehicle followed by superfusate collection. Each superfusate collected was quantitated for CGRP levels by rat-specific CGRP ELISA (Cayman Chemical, Ann Arbor, MI). All experiments were conducted in duplicate with n = six wells per treatment group for a total of approximately 12 wells per group.

For estrogen receptor agonist treatments, cells were pretreated row-wise with either E2-BSA (50 nM), G-1 (10 nM), ICI 182,780 (1 μM), PPT (100 nM), DPN (15 or 100 nM), or vehicle (HBSS or HBSS/DMSO). Superfusate was collected and cells were then stimulation for 15 minutes with 5HT (100 μM) in the continued presence of each drug, superfusate collected, and finally cells were stimulated with capsaicin (50 nM) in the continued presence of 5HT for 15 minutes and superfusate collected. For estrogen receptor antagonist treatments, cells were pretreated row-wise for 15 minutes with either MPP (300 nM), PHTPP (1 μM), or vehicle (HBSS or HBSS/DMSO). Superfusate was collected and cells were treated with E2 (50nM) for 15 minutes followed by superfusate collection. Cells were then stimulated with 5HT (100 μM) in the continued presence of E2 and each drug, superfusate collected, then stimulated with capsaicin (50

nM) followed by supernatant collection. The concentration of each drug was chosen based on previous reports on specificity (see Table 5.1).

Calcium imaging

Trigeminal ganglia (n = 4 rats per 96-well plate) were extracted from adult OVX female rats (< 200 g) immediately following decapitation and cultured as described above, except trypsin was replaced by dispase (2 mg/ml) as per calcium imaging recommendations [55]. Trigeminal ganglia primary cultures were maintained in an incubator for 5 days at 37 °C and 5% CO_2. The assay was performed as per the Fluo-4 calcium imaging kit protocol (ThermoFisher Scientific, Waltham, MA). Briefly, culture media was aspirated from one column at a time (8 wells; A-H), each well was washed 1X with HBSS buffer, and was incubated with 100 µL Fluo-4 AM loading dye. The cells were then incubated at 37 °C and 5% CO_2 for 30 minutes followed by 15 minutes at room temperature. The Fluo-4 was aspirated, cells were washed 1X with HBSS, and 50 µL of Neuro Background Suppressor (proprietary solution) was slowly pipetted into each well. Calcium imaging was recorded using BioTek Cytation 5 plate reader at the GFP optical filter. Baseline fluorescence reading was recorded for 30 sec to 1 minute followed by 50 µL buffer/pretreatment dispense. After one minute buffer/ pretreatment read and 1 minute delay, 50 µL 50 nM CAP was dispensed and reading were recorded over 1–2 min. The change in fluorescent intensity was calculated by subtracting average baseline read from the peak CAP read. All agonists/antagonist concentrations were used as described in the CGRP release assay protocol.

Data analysis

All data were analyzed and graphed with GraphPad Prism software version 8 (GraphPad, San Diego, CA). Orofacial nocifensive behavior data were expressed as mean ± standard error of the mean (SEM) number of forelimb swipes over a 30 min period (6 min bouts) and was analyzed

by repeated measures two-way analysis of variance (ANOVA). CGRP release was reported as mean ± SEM percent of basal CGRP release and were analyzed by unpaired t-test or two-way ANOVA. The Grubb's test (GraphPad Quick Calcs Online, the extreme studentized deviate method; [(mean-value) / standard deviation]) was used to exclude a single outlier within an experimental group if present. Further, 3 animals were removed from the study when environmental factors (significant noise in the animal facility) disrupted behavior testing. Dunnett's test was used for behavior data and Bonferroni's correction was used for CGRP release assays to calculate *a priori* pairwise comparisons when significance was detected by ANOVA.

Results

Steady-state, but not acute, diestrus level E2 treatment significantly reduced peripheral 5HT-evoked orofacial nocifensive behaviors in ovariectomized female rats

Our previous studies reported that 5HT evoked greater pain behaviors in female rats during proestrus and estrus [43; 45]. As E2 levels peak during proestrus but drop during estrus, it remained unclear whether E2 was acting in a pronociceptive or antinociceptive fashion. Here we performed steady-state and acute hormone manipulations in ovariectomized female rats to control the timing and concentration of E2 levels. We define acute exposure as a single subcutaneous injection of E2 given one hour before behavior testing and steady state exposure as continual exposure to the same level of E2 for one week. In the acute E2 exposure experiment, 5HT-evoked nocifensive behaviors were observed at the 7–12 minute time bout (see Figure 5.1A; open bars). Acute exposure to physiological (gray bars) or supraphysiological (closed bars) E2 did not significantly alter 5HT-evoked nocifensive behaviors (see Figure 5.1A; $p > 0.05$). In the steady-state E2 exposure experiment, 5HT-evoked nocifensive behaviors were observed at the 0–18 minute time bouts (see Figure 5.1B; open bars). Steady-state exposure to physiological levels of E2 comparable to diestrus or proestrus significantly attenuated 5HT-evoked nocifensive

behaviors [$F(4, 112) = 8.459$; $p < 0.0001$] at the 0–6 minute time bout (see Figure 5.1B; gray bars; $p \leq 0.05$). 5HT-evoked nocifensive behaviors were unaltered by E2 at the 7–12 minute time bout ($p > 0.05$), but were again attenuated at the 13–18 minute time bout only in the females receiving diestrus-level E2 ($p \leq 0.01$). The highest 5HT-evoked nocifensive behaviors were observed in the animals that received the supraphysiological level E2 (see Figure 5.1B; closed bars; $p > 0.05$).

Several pain pathway related genes are altered in the trigeminal ganglia of E2-treated female rats

Since estrogen is a major hormone involved in multiple gene expression pathways in the cell, and we observed significant differences in the 5HT-evoked pain behaviors in the steady-state E2 experiment, we next determined the corresponding changes in trigeminal ganglia transcriptome of the E2-treated rats from the behavior experiments. Several genes that directly or indirectly play a role in pain signaling were differentially expressed after E2 exposure. Expression of major genes involved in nociception and analgesia was significantly changed in steady-state diestrus (see Figure 5.2A, 5.2B) and proestrus E2 group compared to vehicle (see Figure 5.2C, 5.2D). Similarly, expression of major nociceptive genes was upregulated in the steady-state supraphysiological E2 group as compared to vehicle (see Figure 5.3A, 5.3B) and steady-state diestrus E2 group (see Figure 5.3C, 5.3D). The function of those genes in trigeminal pain signaling and modulation by E2 is listed in Table 5.2.

Estrogen receptor mRNA and $5HT_{2A}$ receptor mRNA are observed in the TRPV1 population of trigeminal sensory neurons in female rats

We report that ERα, ERβ, and GPER mRNA were localized in the trigeminal ganglia of female rats (see Figure 5.4A, E, I). $5HT_{2A}$ receptor mRNA was also observed in small and medium diameter cells (see Figure 5.4B, F, J). TRPV1 ion channel mRNA expression was

predominately found in in distinct small and medium diameter cells, as expected (see Figure 5.4C, G, K). In both males and females there was a subset of TRPV1 mRNA expressing cells that contained both $5HT_{2A}$ receptor mRNA and estrogen receptors, notable ERα (see Figure 5.4D, H, L). Further, TRPV1 mRNA (see Figure 5.5A, D, G, J) and $5HT_{2A}$ receptor mRNA (see Figure 5.5B, E, H, K) was co-expressed in trigeminal ganglia cells (see Figure 5.5C, F, I, L).

Membrane-bound estrogen receptors do not alter serotonergic potentiation of capsaicin-evoked CGRP release from trigeminal sensory neurons

As estrogen receptors are expressed on the 5HT-sensitive TRPV1 population of trigeminal sensory neurons and our previous study reported that E2 enhances serotonergic potentiation of capsaicin-evoked CGRP release from female trigeminal sensory neurons [45], our next series of experiments were designed to determine which E2 receptors are involved in the effects of E2 on 5HT-evoked pain. Pretreatment with 5HT (see Figure 5.6A) or E2-BSA (see Figure 5.6B) alone did not significantly alter CGRP release comparable to vehicle ($p > 0.05$), while capsaicin did evoke significant CGRP release in these cells (see Figure 5.6C; $p \leq 0.05$). There was no effect of the membrane impermeable E2-BSA on 5HT and capsaicin-evoked CGRP release (see Figure 5.6D; $p > 0.05$). Further, there was no effect of pretreatment with the GPER agonist G1 (see Figure 5.7A) or the GPER agonist and classical estrogen receptor antagonist ICI 182,780 (see Figure 5.7B) on 5HT- and capsaicin-evoked CGRP release ($p > 0.05$). The drugs employed to target membrane estrogen receptors did not alter CGRP release alone.

Activation of ERα, but not ERβ, significantly enhances serotonergic potentiation of CGRP release from trigeminal sensory neurons

Since E2 can act on either membrane ERs (GPER) or the classic nuclear ERs (ERα and ERβ), we next tested if selectively targeting either ERα or ERβ would enhance serotonergic potentiation of capsaicin-evoked CGRP release. Treatment with the ERα agonist PPT

significantly increased 5HT potentiated CGRP release (closed bars) as compared to the 5HT and vehicle treatment (open bars) and compared to pretreatment prior to capsaicin stimulation (see Figure 5.8A; $p \leq 0.05$). Pretreatment with 15 nM ERβ agonist DPN did not potentiate capsaicin-evoked CGRP release (see Figure 5.8B; $p > 0.05$). Of note, we initially applied 100 nM DPN which significantly enhanced capsaicin-evoked CGRP release similar to activating ERα (data not shown), however this dosage was reported to be nonspecific and have activity at ERα [34], so we switched to the 15 nM DPN concentration reported here. Pretreatment with the selective ERα antagonist MPP significantly attenuated 5HT potentiated CGRP release as compared to vehicle (see Figure 5.8C; $p \leq 0.05$), while pretreatment with ERβ antagonist PHTPP did not attenuate 5HT potentiated CGRP release (see Figure 5.8D; $p > 0.05$).

5HT+E2 pretreatment enhances CAP-evoked calcium influx

As we found that E2 targeted ERα to enhance serotonergic potentiation of CGRP release, we next tested whether E2 targets ERα to enhance 5HT-evoked or 5HT potentiated capsaicin-evoked calcium influx in trigeminal sensory neurons. Primary cultures of trigeminal ganglia neurons were incubated in Fluo-4 and we observed that capsaicin evoked a transient peak in calcium influx 0–60 seconds following stimulation, as expected (see Figure 5.9A, B). Capsaicin alone evoked significant calcium influx at 30 nM and higher concentrations resulted in desensitization (see Figure 5.9C; $p \leq 0.05$). 5HT alone evoked increasing calcium influx from 1–50 μM, which peaked in significance at 50 μM ($p \leq 0.05$) followed by a drop in calcium influx at 100 μM (see Figure 5.9D). The ERα agonist PPT did not enhance 5HT-evoked calcium influx compared to E2 pretreatment. Also, calcium influx was not attenuated when sensory neurons were pretreated with the ERα antagonist MPP (see Figure 5.9E). Thus far, our data does indicate that pretreatment with E2 prior to 5HT+CAP stimulation may enhance CAP-evoked calcium influx (see Figure 5.9F).

Discussion

Pain conditions with a higher prevalence in women largely include pain conditions that occur in the trigeminal sensory system, which include migraine, tension-type headache, temporomandibular joint disorder (TMD) pain, trigeminal neuralgia, orofacial varicella zoster-associated pain, and burning mouth syndrome. Women also report increased severity and duration of trigeminal pain conditions and often require higher doses of analgesics to treat trigeminal pain compared to men [25,40,70]. Many mechanisms have been postulated to contribute to the female prevalence of pain disorders and sex differences in pain, including sex differences in pain modulation, contributions of sex chromosomes and genetics, modulation by gonadal and peptide hormones, and various psychosocial factors [67,70].

In the present study we elected to study the effects of E2 on peripheral 5HT neuromodulation of trigeminal nociceptors, given that 5HT has been implicated in several pain disorders in the trigeminal system more prevalent in women, including migraine, headache, and TMD pain [29,32,36]. We predicted a nociceptive interaction between E2 and 5HT on female rat trigeminal nociceptors. Here we report that (1) acute injection of E2 did not significantly alter 5HT-evoked orofacial nocifensive behaviors, whereas a steady-state diestrus or proestrus level of E2 significantly attenuated 5HT-evoked orofacial nocifensive behaviors, (2) E2 modulated specific pain genes in a time- and concentration-dependent manner, (3) nuclear and membrane ER mRNA co-express with $5HT_{2A}$ and TRPV1 mRNA in female trigeminal sensory neurons, and (4) activation of ERα, but not ERβ or GPER, significantly enhanced serotonergic potentiation of CGRP release and calcium influx, which was reversed by ERα antagonism.

E2 can modulate the serotonergic system in several ways, including via estrogen response elements, protein-protein interactions, and via membrane estrogen-receptor mediated signaling cascades. E2 modulation of 5HT has been observed as an increase in 5HT levels in the brain [35]

and periphery with increasing E2 levels [1,21], an increase in the expression of the 5HT synthesizing enzymes [3,11], an increase in $5HT_{2A}$ receptor density and binding following hormone replacement therapy in women [83], and capsaicin-evoked proinflammatory peptide and nocifensive behavior are highest when E2 levels peak [56,105]. ERα and ERβ are members of the nuclear receptor superfamily that dimerize on binding to E2 then translocate to the nucleus to control gene transcription by binding to the estrogen response elements (EREs) in the promoter region [60]. On the contrary, membrane bound GPERs act via a rapid G protein-coupled signaling and activate downstream kinases, such as mitogen activated protein kinase (MAPK) and tyrosine kinases [2]. Studies have reported varying roles of the ERs based on the pain model being tested, agonist/antagonist used in the study and CNS vs PNS effects of ERs [17]. In a neuropathic pain model ERβ selective agonists, but not ERα agonists, have been reported to produce antiallodynic effects and alleviate hyperalgesia induced by capsaicin [59,76]. Whereas in inflammatory pain model, ERα increased the activation of NMDA receptors in the spinal cord [95] and activated the MAPK pathway [42], thus facilitating pronociceptive processing.

Evidently, the estrogen receptor subtype activation, the resulting signaling cascade, and concentration of E2 play a vital role in determining the antinociceptive or pronociceptive effects of E2. For instance, Bradshaw et al., reported greater thermal hyperalgesia in proestrus females following a CFA injection in the hindpaw [15], whereas Clemente et al., reported that nociceptive responses post formalin injection were higher in diestrus females [19]. In our previous studies we found that 5HT evoked pain behaviors in the hindpaw [43] and in the vibrissal pad [45] at the highest levels during both, proestrus and estrus while pain behaviors were lowest during diestrus. As we could not discern whether high or low E2 levels were contributing to pain given the similar effect during both proestrus and estrus, in the present study we utilized ovariectomized female rats to control the exposure time and concentration of E2. When animals were injected with

different concentrations of E2 for an acute period (1 hour), we did see an overall rise in nocifensive behaviors at the 7–12 min time bouts across all treatment groups that declines further towards 30 mins, concurrent with our previous behavior studies [45], but the effect was not robust enough to detect significance. Interestingly, when animals were exposed to steady state levels of E2 at varying concentrations, both the 5% E2 (diestrus level) and the 20% E2 (proestrus level) E2 evoked significant attenuation of 5HT-evoked nocifensive behaviors at the first 6 min bout and only the animals that received 5% E2 showed significant reduction in nocifensive behaviors at the 13–18 min bout. Nocifensive behaviors are comparable across all groups at 7–12 min time bout and during the 19–24 and 25–30 min time bouts. Together these data with our previous reports indicate that naturally fluctuating E2 levels during proestrus and estrus enhance pain, while an acute injection of E2 is not sufficient to alter pain. This may be due to neural plasticity (e.g. gonadal hormone-evoked fluctuations in 5HTRs on nociceptors) or differential gene expression occurring during natural fluctuations of the estrous cycle, while an acute injection does not trigger such plasticity. Further, physiological levels of E2 (both diestrus and proestrus) are protective against 5HT-evoked pain, while increasing doses of 5HT contributes to 5HT-evoked pain. This is interesting as the literature has historically used supraphysiological E2 levels to test the effects of E2 on pain and report nociception, while physiological levels may be antinociceptive.

The observed differential effects of E2 on orofacial pain could be attributed, in part, to differential gene expression. To explore this possibility, we extracted the RNA from the trigeminal ganglia of the rats used in the behavior studies and looked for DEGs across the E2-treatment groups. Indeed, genes playing a major role in nociception, inflammatory receptor signaling, and familial migraine were upregulated in supraphysiological E2 group, whereas major analgesic genes such as GABA receptor, cannabinoid receptor, and NMDA receptor subunit were upregulated in diestrus E2 group, concurrent with behavior data.

The observed differential effects of E2 on orofacial pain could also be attributed, in part, to neural plasticity in the expression on 5HT receptors on the TRPV1 population of trigeminal sensory neurons, given that 5HTRs can be increased by E2 exposure. To explore this possibility, we extracted trigeminal ganglia from cycling female rats (and male rats for quantitative comparison) and quantified the levels of co-expression of $5HT_{2A}$ mRNA in the TRPV1 population of trigeminal nociceptors. We focused our efforts on $5HT_{2A}$ mRNA (although other 5HTRs may also be involved) as $5HT_{2A}$ is a major excitatory G protein-coupled receptor previously reported in the TRPV1 population of trigeminal sensory neurons [55] that is capable of sensitizing TRPV1.

E2 can also modulate expression of neuropeptides and other GPCRs in trigeminal ganglia, resulting in differential pain signaling. Puri et al., reported that the expression of the inhibitory neuropeptides declined with diminishing E2 levels over the mouse estrous cycle, thus initiating peripheral sensitization [78]. In addition, in OVX female mice receiving E2 replacement for 7 days, E2 was reported to regulate proteins involved in G protein signaling, indicating a regulatory role of E2 in modulating peripheral pain processing [102]. This supports the differences we see in nocifensive behaviors, gene expression, and co-expression of receptors over the estrus cycle.

Treatment with ERβ agonist DPN has been reported to increase the expression of tryptophan hydroxylase-2 (Tph2) in the brain via estrogen response elements (ERE) present in the Tph2 promoter [38,88]. ERs can also act via the non-classical pathway to activate ERE. This mechanism involves heterodimerization of the classical ERα or ERβ receptors with transcription factors such as NF-κB that can bind to the EREs and alter gene transcription [7]. One specific study by Wissink et al., reported that ERα can synergistically act with NF-κB to activate the $5HT_{1A}$ promoter [104]. Furthermore, E2 inhibits the expression of serotonin reuptake transporter

(SERT) in the CNS, thus increasing the availability of 5HT in synaptic spaces [12]. Apart from the 5HT system, E2 can also upregulate TRPV1 mRNA in the female trigeminal ganglia in a dose dependent manner [105].

To study the functional output of the modulatory role of E2 on serotonergic potentiation in the trigeminal ganglia, we also performed an array of *ex vivo* experiments wherein we treated primary cultures of trigeminal sensory neurons with ER agonists and antagonists. We have previously reported that E2 pretreatment can significantly enhance serotonergic potentiation of CGRP release from cultured trigeminal sensory neurons [45]. In addition, Rowan et al., has reported that E2 can enhance bradykinin responses in cultured trigeminal ganglia neurons [81]. Here we sought to elucidate the specific ER involved in the observed serotonergic potentiation of CGRP release. Pretreatment with E2-BSA (membrane impermeable E2), G1 (selective GPER agonist), or ICI 182,780 (GPER agonist, ERα/β antagonist) did not mimic E2 enhancement of serotonergic potentiation of CGRP release, indicating the potentiation activity is independent of membrane bound ERs. When treated with the ERα agonist PPT there was a significant increase in CGRP release comparable to our previous reports of E2 enhancement of serotonergic potentiation of CGRP release. Treatment with the ERβ agonist DPN had no effect unless a higher, non-specific concentration was utilized that also activated ERα. Further, treatment with the ERα antagonist MPP, but not the ERβ antagonist PHTPP, reversed the enhancement of CGRP release indicating that ERα activation underlies the pronociceptive effects of E2 on trigeminal sensory neurons.

Overall, we present evidence that the timing and concentration of E2 in female rats determines whether E2 contributes to or attenuates 5HT-evoked orofacial pain and that activation of ERα, but not ERβ, is responsible for exacerbation of serotonergic potentiation of trigeminal pain signaling. Further mechanisms by which E2 modulates trigeminal sensory neurons include

altering gene expression of known pain genes in the female trigeminal ganglia transcriptome and inducing neural plasticity in the major excitatory 5HT receptors present on a subpopulation of nociceptive trigeminal ganglia neurons. These data contribute to the understanding of the complex and paradoxical role of E2 on orofacial pain and should be considered when treating trigeminal pain disorders in women following menarche, prior to menopause, and when electing estrogen replacement therapy.

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Figures and Tables

Pharmacologic	Target	Company	Cat. #	Concentration	Ref.
E2-BSA	Membrane-impermeable E2	Sigma–Aldrich	E5630	50 nM	[81]
G-1	GPER agonist	Cayman Chemical	10008933	10 nM	[13,79]
ICI 182,780	ER antagonist; GPER agonist	Sigma–Aldrich	5310420001	1 μM	[82]
PPT	ERα agonist	Sigma–Aldrich	H6036	100 nM	[18,51,82]
DPN	ERβ agonist	Sigma–Aldrich	H5915	15 nM	[18,65,82,100]
MPP	ERα antagonist	Sigma–Aldrich	M7068	300 nM	[82,92]
PHTPP	ERβ antagonist	Sigma–Aldrich	SML1355	1 μM	[18,49]

Table 5.1: **Specifications and concentrations of the estrogen receptor (ER) agonists and antagonists applied to trigeminal ganglia neuron primary cultures. E2-BSA:** β-Estradiol 6-(O-carboxymethyl)oxime conjugated to bovine serum albumin (BSA); **G-1:** *rel*-1-[4-(6-bromo-1,3-benzodioxol-5-yl)-3aR,4S,5,9bS-tetrahydro-3H-cyclopenta[c]quinolin-8-yl]-ethanone; **ICI 182,780**: 7α,17β-[9-[(4,4,5,5,5 Pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol; **PPT**: 1,3,5-Tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole; **DPN**: 2,3-Bis(4-hydroxyphenyl)propionitrile; **MPP**: MPP Dihydrochloride; **PHTPP**: 4-[2-Phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]-pyrimidin-3-yl]phenol.

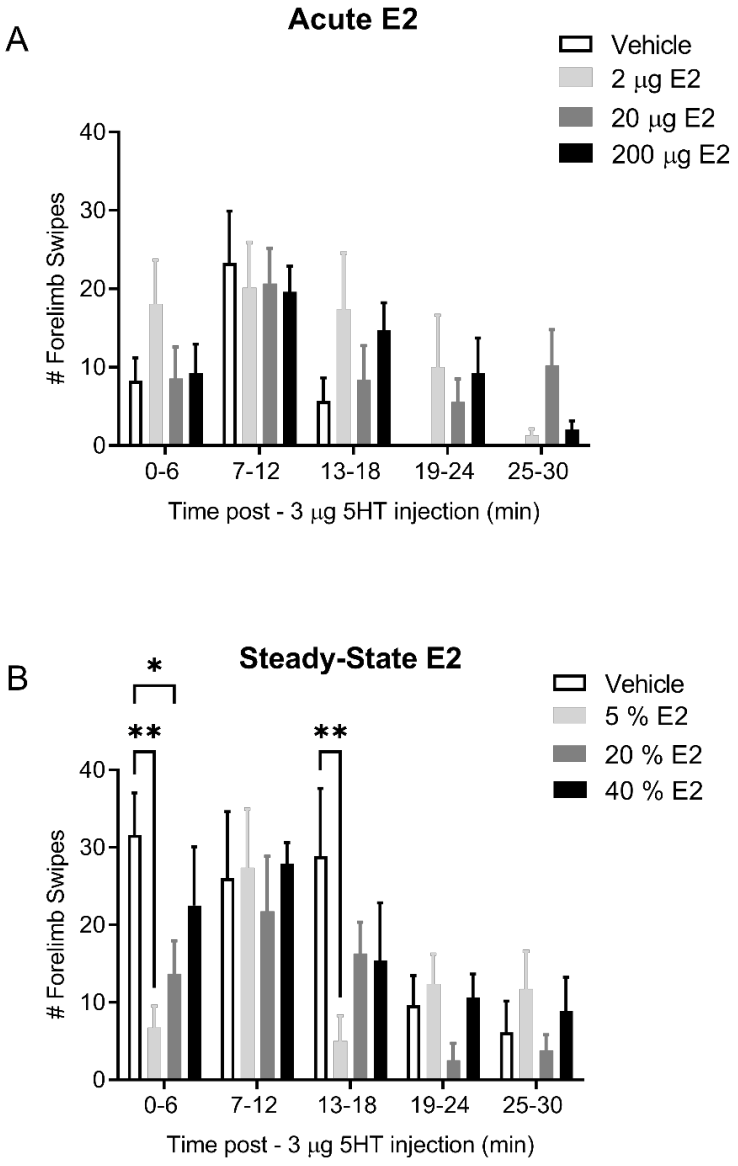
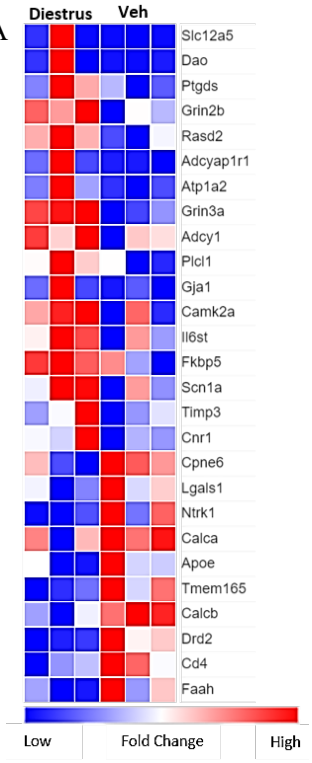


Figure 5.1. **Steady-state physiological levels of E2, but not acute E2 treatment, attenuates 5HT-evoked orofacial pain behaviors**. 5HT-evoked orofacial pain behaviors were not significantly different in OVX females that received 2 μg E2 (light gray bars), 20 μg E2 (dark gray bars), or 200 μg E2 (closed bars) one hour prior to receiving 5HT injection as compared to vehicle control (open bars) (A). 5HT-evoked pain behaviors were significantly reduced in OVX females that were administered with steady-state physiological levels of 5% E2 (light gray bars) and 20% E2 capsules (dark grey bars) compared to vehicle control (open bars) at 0–6 min (B). Attenuation was also reported in the 5% E2 group at 13–18 min time bout. Attenuation was not observed in animals that received 40% E2 capsules (closed bars). *Denotes significant number of forelimb swipes compared to vehicle with significance in pairwise comparisons tested at $p \leq 0.05$.

A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Grin3a
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Gja1
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Fkbp5
Scn1a
Timp3
Cnr1
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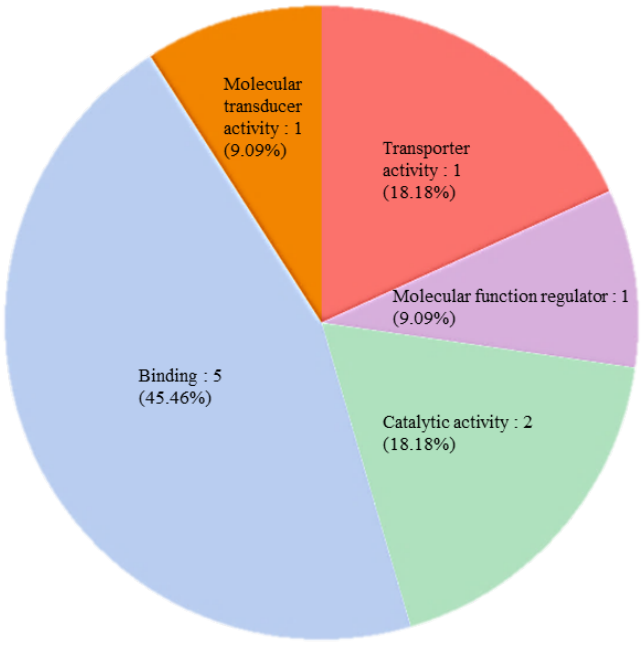
B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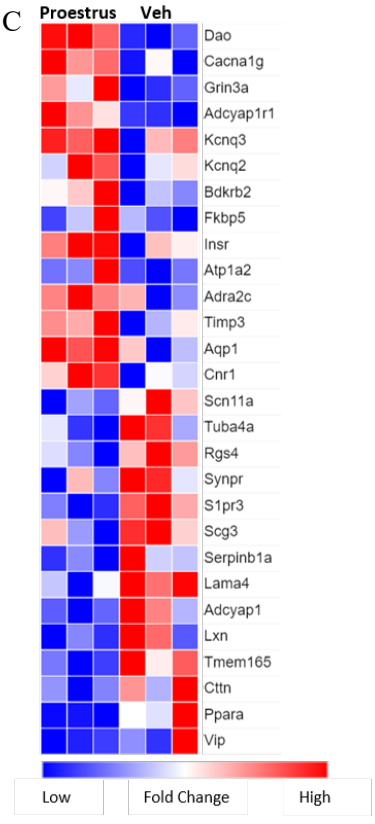
Transporter activity : 1 (18.18%)
Molecular function regulator : 1 (9.09%)
Catalytic activity : 2 (18.18%)
Binding : 5 (45.46%)

C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Synpr
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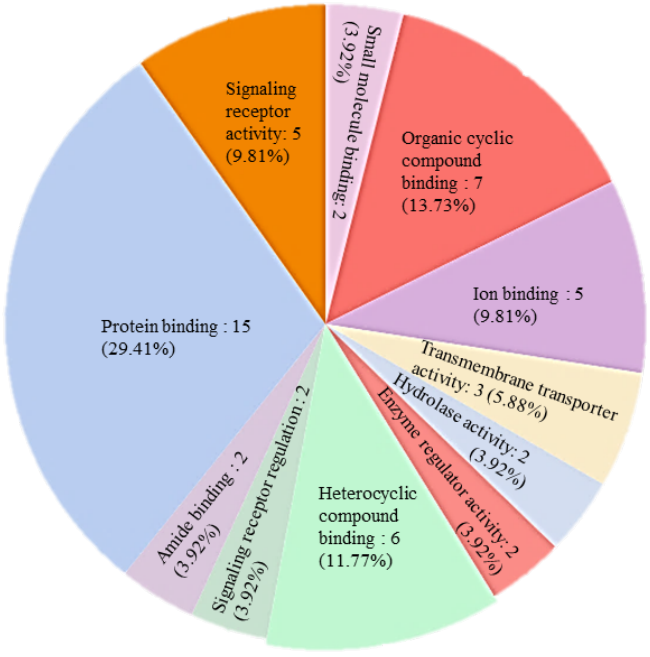
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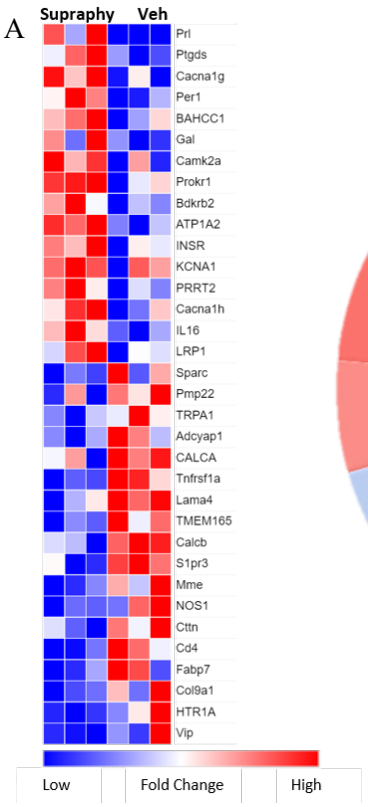
Organic cyclic compound binding : 7 (13.73%)
Ion binding : 5 (9.81%)
Transmembrane transporter activity: 3 (5.88%)
Hydrolase activity: 2 (3.92%)
Enzyme regulator activity: 2 (3.92%)
Heterocyclic compound binding : 6 (11.77%)
Signaling receptor regulation : 2 (3.92%)
Amide binding : 2 (3.92%)
Protein binding : 15 (29.41%)
Signaling receptor activity: 5 (9.81%)

Figure 5.2. Several genes related to trigeminal pain processing are altered in the steady-state diestrus and proestrus E2 groups. Differentially expressed genes (DEGs) in steady-state diestrus E2 group compared to vehicle (Veh; A) and molecular function analysis of the DEGs (B). Differentially expressed genes (DEGs) in steady-state proestrus E2 group compared to vehicle (Veh; C) and molecular function analysis of the DEGs (D). Blue=downregulation; red=upregulation. Only the genes that significantly changed ($p < 0.05$) are shown.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IL16
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Lama4
TMEM165
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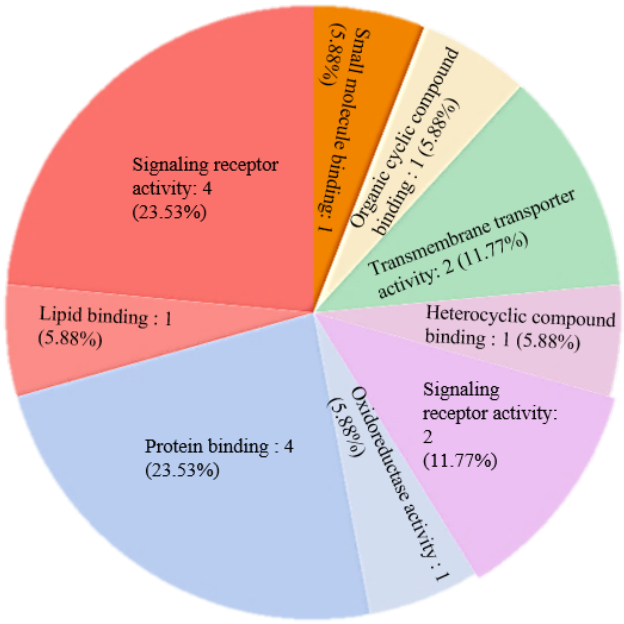
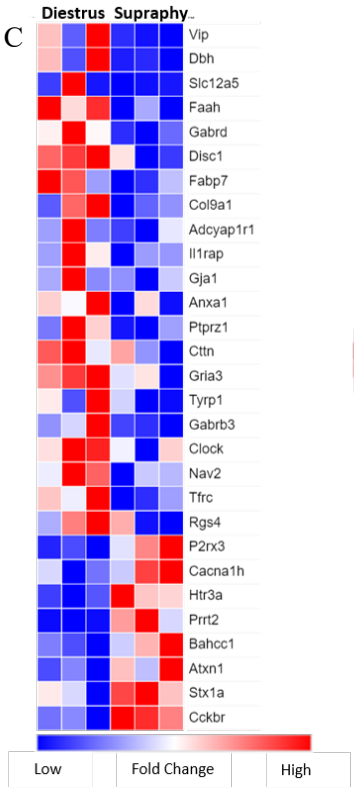

B
Small molecule binding: 1 (5.88%)
Organic cyclic compound binding : 1 (5.88%)
Transmembrane transporter activity: 2 (11.77%)
Heterocyclic compound binding : 1 (5.88%)
Signaling receptor activity: 2 (11.77%)
Oxidoreductase activity : 1 (5.88%)
Protein binding : 4 (23.53%)
Lipid binding : 1 (5.88%)
Signaling receptor activity: 4 (23.53%)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Gabrb3
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Rgs4
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Cacna1h
Htr3a
Prrt2
Bahcc1
Atxn1
Stx1a
Cckbr
Low
Fold Change
High

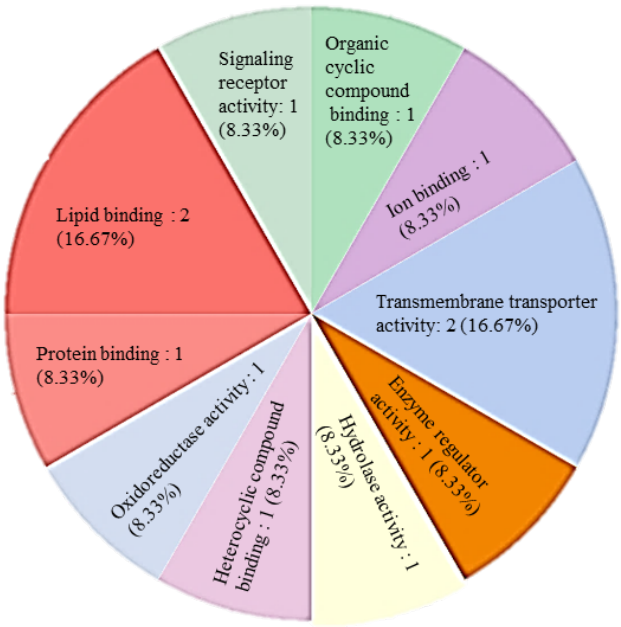
D
Signaling receptor activity: 1 (8.33%)
Organic cyclic compound binding : 1 (8.33%)
Ion binding : 1 (8.33%)
Transmembrane transporter activity: 2 (16.67%)
Enzyme regulator activity : 1 (8.33%)
Hydrolase activity : 1 (8.33%)
Heterocyclic compound binding : 1 (8.33%)
Oxidoreductase activity : 1 (8.33%)
Protein binding : 1 (8.33%)
Lipid binding : 2 (16.67%)

Figure 5.3. Several genes related to trigeminal pain processing are altered in the steady-state supraphysiological E2 group. Differentially expressed genes (DEGs) in steady-state supraphysiological (Supraphy) E2 group compared to vehicle (Veh; A) and molecular function analysis of the DEGs (B). Differentially expressed genes (DEGs) in steady-state supraphysiological E2 group compared to steady-state diestrus E2 (C) and molecular function analysis of the DEGs (D). Blue=downregulation; red=upregulation. Only the genes that significantly changed ($p < 0.05$) are shown.

Gene symbol	Gene Name	Function	Comparison	Ref
Dao	Diamine oxidase	Scavenging extracellular histamine after mediator release	Pro > Veh Di > Veh	[85]
Bdkrb2	Bradykinin 2 receptor	Proinflammatory; pronociceptive	Pro > Veh Sp > Veh	[81]
Cacna1g	Calcium channel subunit alpha 1G	Voltage-gated T-type calcium channel (Cav3.1)	Sp > Veh Pro > Veh	[14]
Cacna1h	Calcium channel subunit alpha 1H	Voltage-gated T-type calcium channel (Cav3.2)	Sp > Veh Sp > Di	[14]
Prrt2	Prolein rich transmembrane protein	Negative regulator of Nav1.2/1.6 Complex disorders with migraine; type 4 familial hemiplegic migraine; in PRF pain genes	Sp > Veh Sp > Di	[30,48]
Grin3a	NMDA receptor subunit 3a	Ionotropic glutamate receptor; involved in pain inhibition	Di > Veh Pro > Veh	[68]
Gabrd	GABA receptor delta	Target for analgesia	Di > Sp	[58]
Adcyap1r1	Pituitary adenylate cyclase-activating polypeptide (PACAP) type 1 receptor (PAC1)	Involved in migraine Regulated by stress and E2	Pro > Veh Di > Veh Di > Sp	[46,110]
Adcyap1	Pituitary adenylate cyclase-activating polypeptide 1 (PACAP)	Involved in migraine Neuropathic pain onset	Pro < Veh Sp < Veh	[26,110]
Vip	Vasoactive intestinal polypeptide	Same superfamily as PACAP (glucagon/secretin); neuropathic pain maintenance	Pro < Veh Sp < Veh Sp < Di	[26]
Prl	prolactin	Female-specific hyperalgesia Modulated by E2	Sp > Veh	[73,99]
Calca	Calcitonin gene-related peptide alpha	Involved in migraine	Di < Veh Sp < Veh	[110]
Calcb	Calcitonin gene-related peptide beta	Involved in migraine	Di < Veh Sp < Veh	[110]
Fkbp5	FK506 binding proteins	Co-chaperone of Hsp90 of steroid receptor complex (stabilizing and shuttling) Linked to stress disorders	Pro > Veh Di > Veh	[96,108]

Atp1a2	Na+/K+ pump subunit alpha 2	Involved in familial migraine; E2 rescues age-related drop in ATPase; in PRF pain genes	Pro > Veh Di > Veh Sp > Veh	[47,48]
Timp3	Metalloproteinase inhibitor 3	Protects against cartilage degradation	Pro > Veh Di > Veh	[50]
Cnr1	Cannabinoid receptor 1	Peripheral cannabinoid receptor; peripheral analgesia	Pro > Veh Di > Veh	[106]
Faah	Fatty acid amide hydrolase	Inhibition reduces pain, linked to migraine; reduced FAAH leads to increased cannabinoid and 5HT activity; mutation leads to pain insensitivity; in PRF pain genes	Di < Veh Di > Sp	[33,69]
Rgs4	Regulator of G protein signaling (RGS) 4	Regulatory molecule that acts as a GTPase activating protein (GAP). Can deactivate Gi and Gq. Maintain chronic pain.	Pro < Veh Di > Sp	[6]
S1pr3	Sphingosine-1-phosphate receptor 3	Acute mechanonociception via KCNQ2/3 (Pro > Veh)	Pro < Veh Sp < Veh	[37]
Cttn	Cortactin	Actin rearrangement, regulates vascular permeability, works with S1P	Pro < Veh Sp < Veh	[31]
Ptgds	Prostaglandin D2 synthase (PGD2)	Proinflammatory, produced by neuron, glia, immune cells	Di > Veh Sp > Veh	[41]
Insr	Insulin receptor	Insulin deficiency contributes to diabetic neuropathy	Pro > Veh Sp > Veh	[109]
Lama4	Laminin subunit alpha-4	Heat pain sensitivity	Pro < Veh Sp < Veh	[103]
Tmem165	Transmembrane protein 165	Intracellular calcium transporter	Pro < Veh Di < Veh	[14]
Camk2a	Calcium/calmodulin dependent protein kinase II alpha	Serine/threonine protein kinase; loss contributes to switch from mechanoreception to pain	Di > Veh Sp > Veh	[107]
Cd4	CD4 molecule	Membrane glycoprotein of immune cells (T cells, B cells, macrophages)	Di < Veh Sp < Veh	[62]

Bahcc1	BAH domain and coiled-coil containing 1	Unknown relevance to nociception, but was reported as a SNP in only females with TMD	Sp > Veh Sp > Di	[84]
Fabp7	Fatty acid binding protein 7	Increased in DRG with nerve injury; related to regeneration and PPAR signaling	Sp < Veh Sp < Di	[5,24]
Col9a1	Collagen type IX alpha chain	Mutation leads to painful stiff joints	Sp < Veh Sp < Di	[16]
Disc1	Disrupted in Schizophrenia (DISC1) scaffold protein	Deletion reduces thermal pain sensitivity	Di > Sp	[98]
Anxa1	Annexin A1	Anti-inflammatory; antinociceptive	Di > Sp	[75]
Clock	Circadian regulator	Dimerizes with bmal; target to reduce OA pain	Di > Sp	[23]
Nav2	Neuron navigator, unc-53 homolog	Mutants have impaired sensory function; diminished TG and DRG sensory neurons	Di > Sp	[64]
P2rx3	Purinergic receptor P2X3	Orofacial pain (TMD) and burning mouth syndrome E2 reduces spinal P2X3 E2 attenuates P2X3 in DRG	Sp > Di	[9,57,80,87]
Cckbr	Cholecystokinin B receptor	Anti-opioid activity by heterodimerizing with MOR; E2 increases in MCF-7 cells	Sp > Di	[89]
Htr3A	5HT3A receptor	Ionotropic, pain	Sp > Di	[27]

Table 5.2. Differentially expressed genes following E2 treatment. List of known pain genes that were differentially expressed following steady-state treatment with diestrus-level E2 (Di), proestrus level E2 (Pro), supraphysiological-level E2 (Sp) E2, or vehicle (Veh) in ovariectomized female rats.

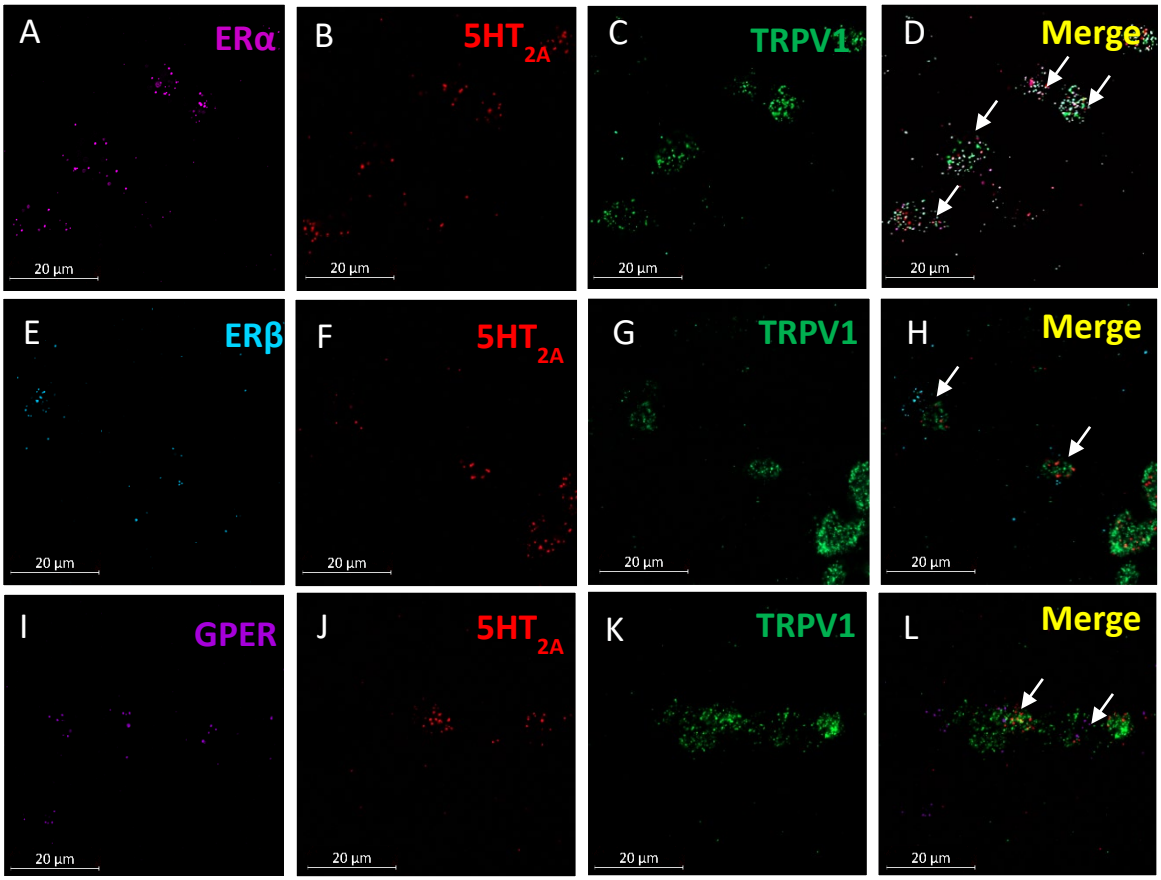

Figure 5.4. ERα, ERβ, and GPER mRNA along with $5HT_{2A}$ mRNA coexpress with TRPV1 mRNA in the female rat trigeminal ganglia. Panels A–D display representative images of the expression of ERα mRNA (violet; A) and $5HT_{2A}$ mRNA (red; B) with TRPV1 ion channel mRNA (green; C) in the trigeminal ganglia (merge; D). Panels E–F display representative images of the expression of ERβ mRNA (blue; E) and $5HT_{2A}$ mRNA (red; F) with TRPV1 ion channel mRNA (green; G) in the trigeminal ganglia (merge; H). Panels I–L depict representative images of the expression of GPER mRNA (purple; I) and $5HT_{2A}$ mRNA (red; J) with TRPV1 ion channel mRNA (green; K) in the trigeminal ganglia (merge; L). Arrows indicate trigeminal ganglia cells co-expressing estrogen receptors and $5HT_{2A}$ receptor mRNA with TRPV1 ion channel mRNA.

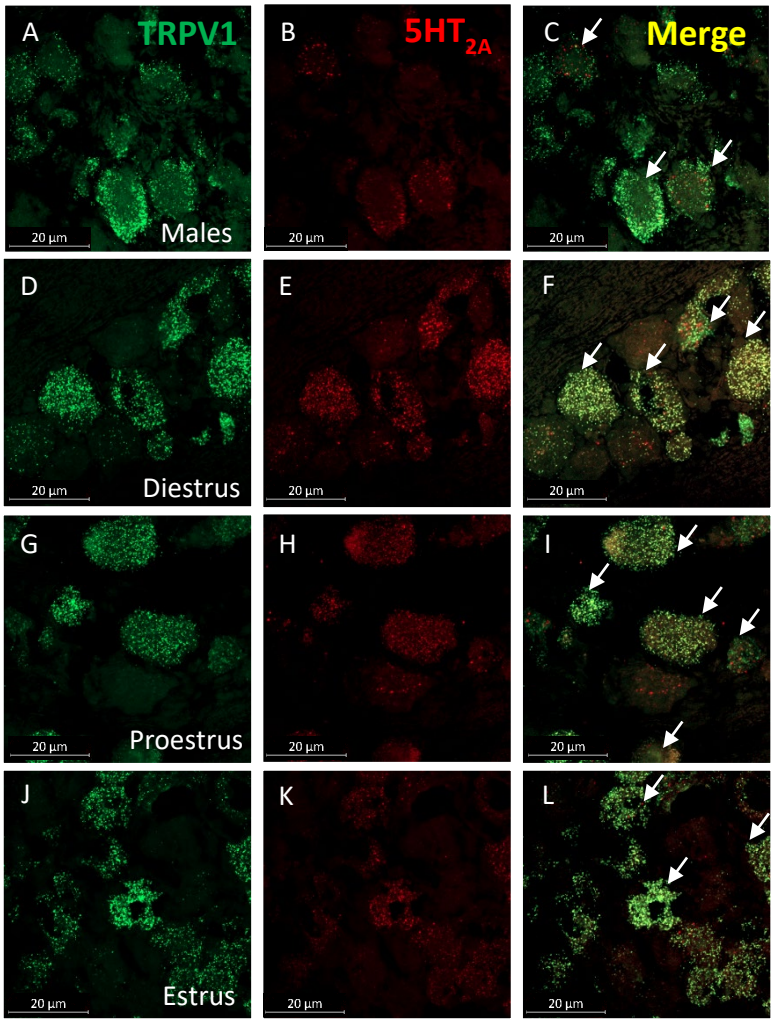

Figure 5.5. $5HT_{2A}$ and TRPV1 mRNA are co-expressed in male and female trigeminal ganglia. Representative images of the expression of TRPV1 ion channel mRNA (green) and $5HT_{2A}$ receptor mRNA (red) in the trigeminal ganglia of male (A–C) and cycling female rats across the stages of the estrous cycle [diestrus (D–F), proestrus (G–I), and estrus (J–L)]. The first column illustrates TRPV1 ion channel mRNA expression, the second column illustrates $5HT_{2A}$ receptor mRNA expression, the third column is an overlay of TRPV1 ion channel mRNA and $5HT_{2A}$ receptor mRNA. Arrows indicate trigeminal ganglia cells co-expressing TRPV1 ion channel mRNA and $5HT_{2A}$ receptor mRNA (yellow).

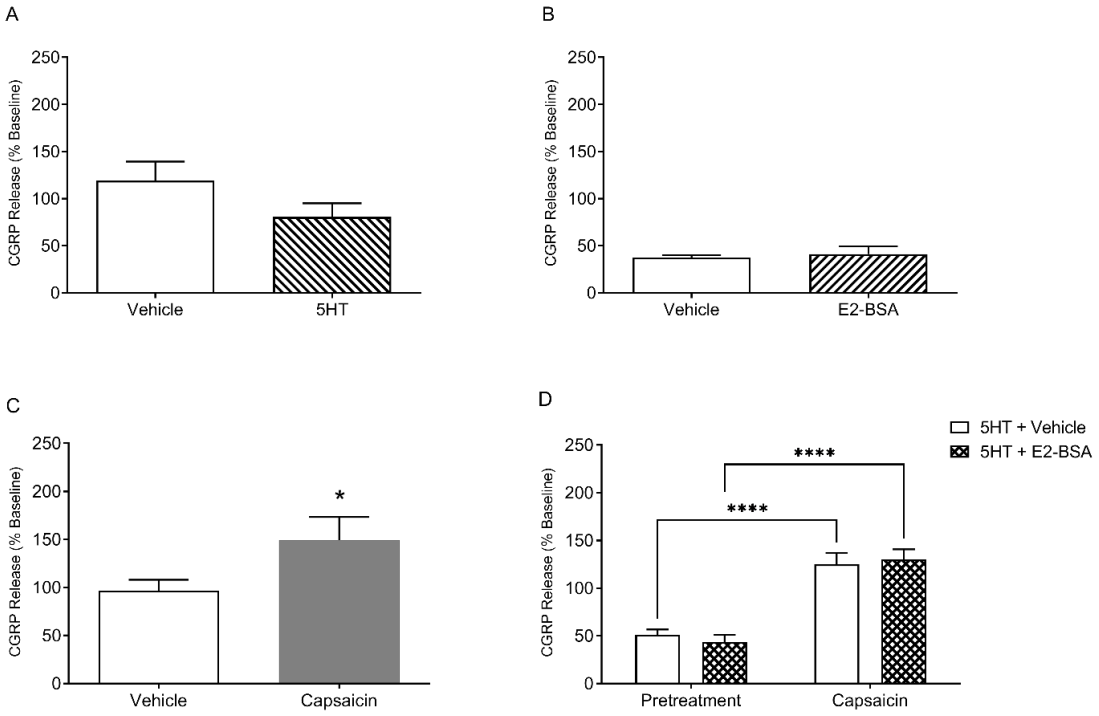

Figure 5.6. E2-BSA does not increase serotonergic potentiation of capsaicin-evoked CGRP release. CGRP release was comparable to vehicle (open bar) with 5HT only (right diagonals) pretreatment (A) and E2-BSA only (left diagonals) pretreatment (B) as observed by percent change in baseline. Capsaicin (grey bar) treatment significantly increased CGRP release compared to vehicle as observed by percent change in baseline (C). E2-BSA+5HT (hatched bar) pretreatment did not significantly enhance capsaicin-evoked CGRP release as compared to the vehicle and 5HT only treatment (D). *Denotes a significant increase in CGRP release compared to vehicle with significance in pairwise comparisons tested at $p \leq 0.05$.

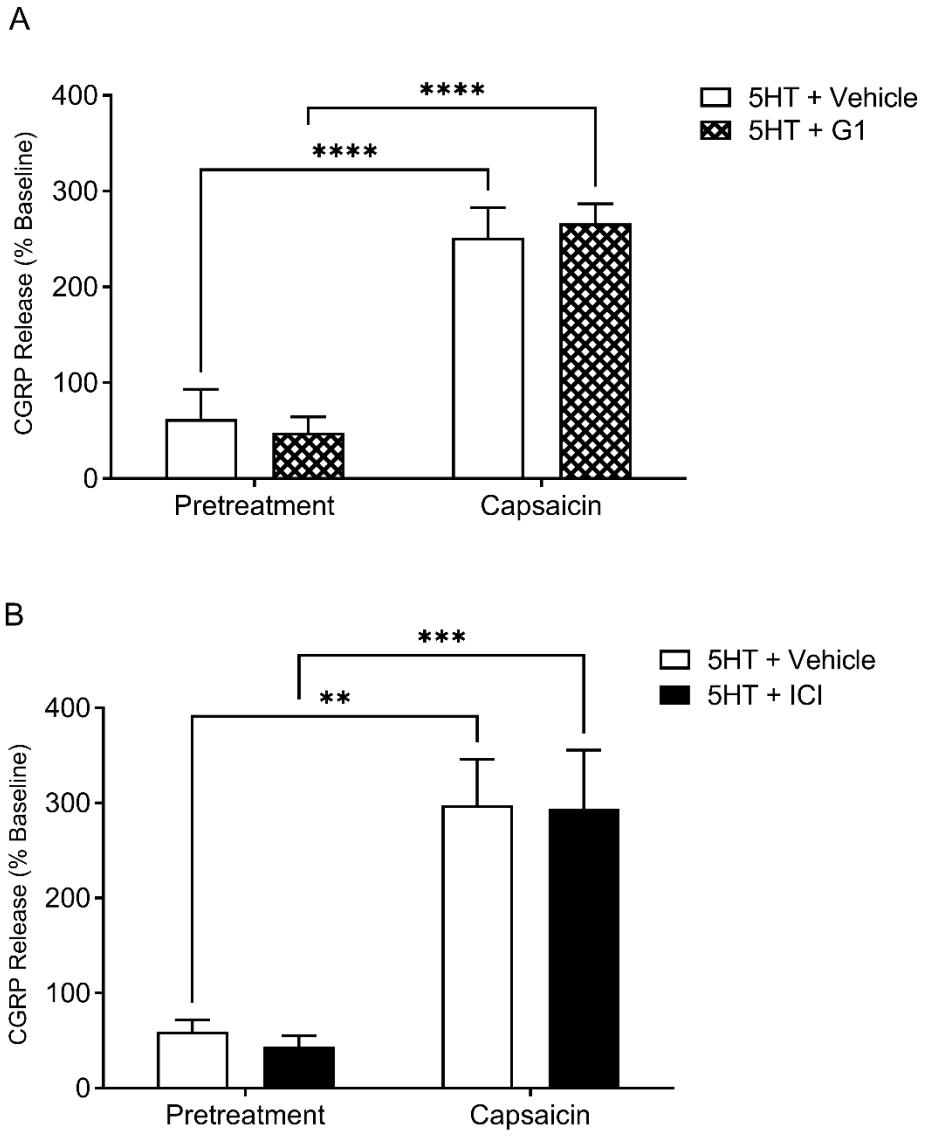

Figure 5.7. Activating the membrane bound ER (GPER) does not increase serotonergic potentiation of capsaicin-evoked CGRP release. G1 (GPER agonist**;** hatched bar) pretreatment (A) and ICI 182,780 (GPER agonist / ER antagonist; closed bar) pretreatment (B) does not enhance capsaicin-evoked CGRP release compared to 5HT + Vehicle only (open bar) treatment. *Denotes a significant increase in CGRP release compared to vehicle with significance in pairwise comparisons tested at $p \leq 0.05$.

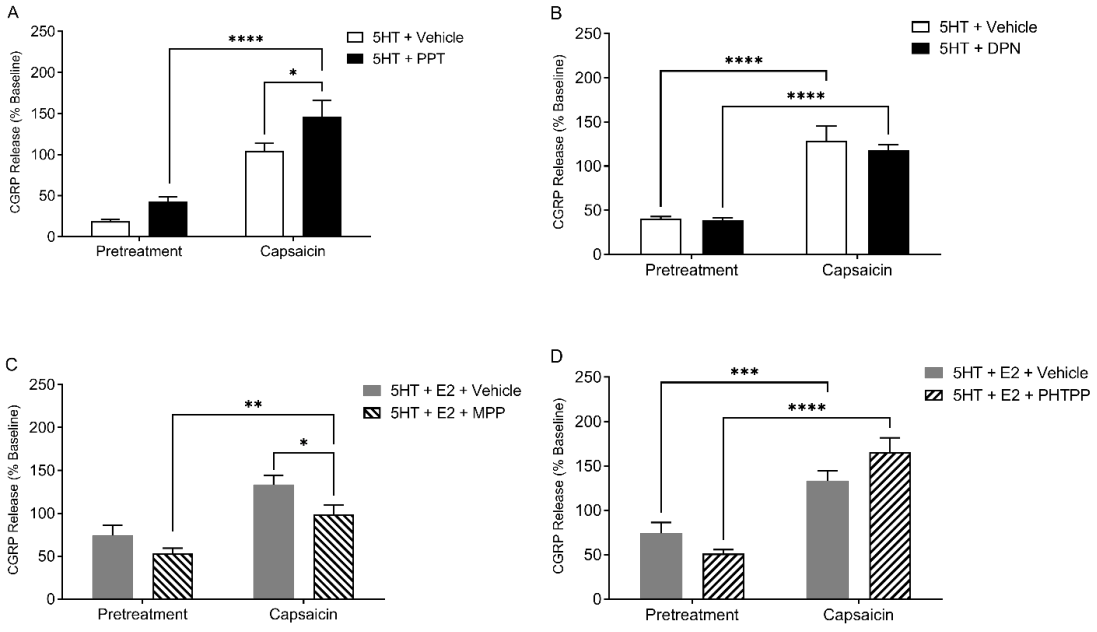

Figure 5.8. ERα plays a significant role in modulating serotonergic potentiation of capsaicin-evoked CGRP release. PPT (ERα agonist; closed bar) pretreatment (A), but not DPN (ERβ agonist; closed bar) pretreatment (B) significantly enhanced the serotonergic potentiation of capsaicin-evoked CGRP release as compared to 5HT+Vehicle control group (open bars). MPP+E2 (ERα antagonist; right diagonals) pretreatment (C), but not PHTPP+E2 (ERβ antagonist; left diagonals) pretreatment (D) significantly attenuated capsaicin-evoked CGRP release compared to 5HT + E2 (grey bar) treatment. *Denotes a significant increase in CGRP release compared to vehicle with significance in pairwise comparisons tested at $p \leq 0.05$.

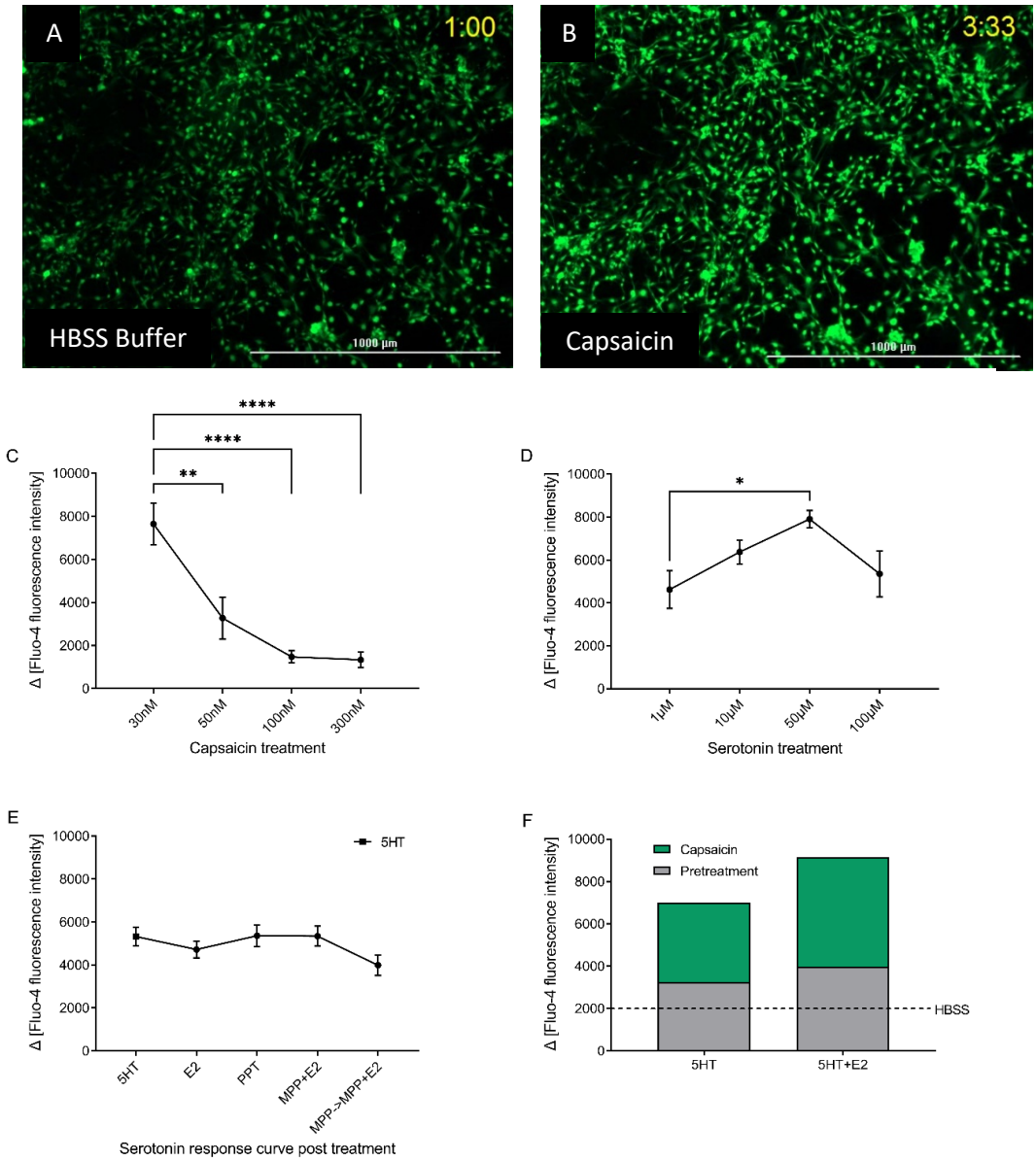

Figure 5.9. E2 plays a significant role in modulating serotonergic potentiation of capsaicin-evoked calcium influx. Representative 4X fluorescent images of cultured trigeminal sensory neurons in presence of buffer treatment (A) and TRPV1 agonist capsaicin (B). 30 nM capsaicin triggered significantly higher calcium influx as compared to higher capsaicin concentrations where desensitization occurred (C), whereas 50 µM 5HT triggered significantly higher calcium influx as compared to 1, 10, and 100 µM 5HT (D). The ERα agonist PPT did not enhance 5HT-evoked calcium influx compared to E2 pretreatment (E) E2 pretreatment increases CAP-evoked calcium influx. *Denotes a significant increase in CGRP release compared to vehicle with significance in pairwise comparisons tested at $p \leq 0.05$.

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CHAPTER VI

CONCLUSIONS AND FUTURE DIRECTIONS

Pain conditions primarily affecting women commonly occur in the trigeminal sensory system and our research provides evidence across multiple lines of investigation that gonadal hormonal modulation of the serotonergic system plays a vital role in trigeminal pain modulation. This dissertation provides a comprehensive behavioral, neuroanatomical, cellular, and genetic analysis of the contribution of gonadal hormone modulation of one inflammatory pain mechanism in female rats, serotonergic potentiation of pain signaling in trigeminal nociceptors. Overall, we provide evidence that 5HT evokes more pain in two distinct sensory neuron populations, the dorsal root ganglia and the trigeminal ganglia, and underlying this sexually dimorphic behavior is a complex pain mechanism that is modulated by estrogen.

Summary of Findings

In our initial studies, reported in Chapter II, when 5HT was injected into the rat hindpaw (involving sensory neurons of the dorsal root ganglia), we reported that 5HT evoked significantly higher sensitivity to noxious heat and non-noxious touch in intact, cycling female rats that were in proestrus or estrus as compared to females in diestrus, ovariectomized females, or males. 5HT evoked pain behaviors within ~5–10 minutes, which peak at ~15 minutes and resolve at around ~30 minutes, though edema can last up to ~60 minutes, as previously reported (Loyd et al., 2012). We did not observe any sex- or estrous cycle-dependent differences in 5HT-evoked edema in the hindpaw or 5HT content in the interstitial fluid of an inflamed rat hindpaw. The proinflammatory and pronociceptive effect of 5HT can be attributed to, at least, the $5HT_{2A}$ receptor as pretreatment

with a $5HT_{2A}$ receptor-selective antagonist attenuated 5HT-evoked pain behaviors and edema in cycling female as well as male rats.

Given that many female prevalent pain conditions occur in the trigeminal system, we extended our studies to testing at the vibrissal pad of the rat, a location of dense innervation by trigeminal nociceptors. Reported in Chapter III, we are the first to report that 5HT evokes significant orofacial nocifensive behaviors in female rats during proestrus and estrus (rapid E2 fluctuations) and in ovariectomized females as compared to vehicle injections. Females in diestrus (steady-state low E2) and males displayed some increases in orofacial nocifensive behaviors but they were not robust enough to reach significance by three-way ANOVA. These data concur with the estrous-cycle and sex-specific effects observed in the rat hindpaw as reported in Chapter II. We also tested the effects of administering 5HT with capsaicin, as 5HT can sensitize TRPV1, and we found that a lower dose of 5HT can evoke nocifensive pain behaviors when capsaicin is also present, but only in females in proestrus, whereas higher doses of 5HT can exacerbate capsaicin-evoked pain behaviors in estrus females and males. In contrast, blocking the $5HT_{2A}$ receptor attenuates 5HT-evoked orofacial pain behaviors in proestrus and estrus females and not males. Of interest, the basal levels of 5HT are higher in interstitial fluid from the vibrissal pad of cycling females as compared to males. Increased plasma 5HT levels are also reported in women receiving fluoxetine (selective serotonin reuptake inhibitor) for treatment of depression (Blardi et al., 2002).

As we observed enhanced pain behaviors during phases of peaking (proestrus) and declining (estrus) E2, we extracted trigeminal ganglia from ovariectomized rats to gain control of the E2 level presented to the trigeminal sensory neurons to determine whether E2 is pronociceptive or antinociceptive. We found that E2 significantly enhanced the pronociceptive effects of 5HT on the TRPV1 population of *ex vivo* trigeminal sensory neurons. We interpret this

as high E2 is pronociceptive on the serotonergic pain mechanism in an environment where only E2, 5HT, and capsaicin are present. This does not account for the pain behaviors we observed during estrus, unless neurobiological plasticity is occurring in the trigeminal ganglia that persists into estrus. Alternatively, in the behaving rat there are other cell types present, including immune cells that can alter E2 modulation of the trigeminal nociceptors. We attempted to address these two possibilities in Chapter IV, which are discussed shortly.

Together, these data (Chapter II and Chapter III results) indicate that while dorsal root ganglia nociceptors and trigeminal ganglia nociceptors are more sensitive to the pronociceptive effects of 5HT, likely acting via excitatory $5HT_{2A}$ receptors on nociceptors, in females when gonadal hormones are rapidly peaking and declining (see Table 6.1), the trigeminal nociceptors appear to be more easily sensitized at lower 5HT doses, which may underlie the greater prevalence of orofacial pain disorders in women where 5HT is a component of the pathophysiology, such as migraine and temporomandibular joint disorder. Our data also highlights the distinction between the two sensory ganglia and emphasizes the importance of understanding molecular mechanisms underlying pain signaling in TG and DRG. Translational profiling and colocalization studies have confirmed important differences between TG and DRG system. Of importance, CGRP and TRPV1 colocalization was significantly higher in TG than DRG, and capsaicin induced behavioral responses were reported to be higher in TG (Megat et al., 2019; Price & Flores, 2007). Table 6.1 summarizes the 5HT-evoked behaviors reported in this dissertation across the TG and DRG system with emphasis on the excitatory $5HT_{2A}$ receptor.

Since previous studies have reported the role of excitatory $5HT_{2A}$ and $5HT_{3A}$ receptors in enhancing TRPV1 function (Loyd et al., 2011), in Chapter IV, we determined whether $5HT_{3A}$, a ligand-gated ion channel, plays a role in 5HT-evoked pain behaviors. We report that blocking the $5HT_{3A}$ receptor does not attenuate 5HT-evoked orofacial pain behaviors or *ex vivo* capsaicin-

evoked CGRP release. In support, enhancement of TRPV1 function by 5HT was not attenuated by $5HT_3$ antagonism in mouse colon sensory neurons (Sugiuar et al., 2004), implicating involvement of metabotropic 5HT receptors. While studies have reported the role of $5HT_3$ in modulating serotonergic pain mechanisms, the antagonist concentration, treatment regime, and pain model used were different than our study, which may account for the differences (Ernberg et al., 2000a; Sung et al., 2008). Of interest might be to study the $5HT_4$ receptor in trigeminal pain processing. Studies have shown that blocking the $5HT_4$ receptor attenuates capsaicin-evoked currents in mouse DRG neurons (Sugiuar et al., 2004), and 5HT-induced hyperexcitability in rat DRG neurons (Lopez et al., 2021). In synopsis, we report a major role of $5HT_{2A}$, but not $5HT_3$ in 5HT-evoked pain behaviors during the phases when E2 rapidly fluctuates. We also report increased 5HT levels in intact cycling rats as compared to males.

Thus, in Chapter V, we focused more on the role of estrogen concentration and exposure time and nuclear/ membrane-bound estrogen receptors since E2 has been reported to play a major role in modulating serotonin's pronociceptive effects on trigeminal pain processing (Kaur et al., 2021b). We report that a steady-state low dose of diestrus level E2 was protective against 5HT-evoked pain whereas a steady-state supraphysiological level E2 exacerbated 5HT-evoked pain. In support, Kramer & Bellinger (2009) have also reported that a low, continual dose of E2 appears to be antinociceptive in TMJ pain whereas a high E2 dose is pronociceptive. Additionally, TMD pain decreases post-menopause and exacerbates in women undergoing hormone replacement therapy (Craft, 2007). Also, high plasma levels of E2 are reported in women who report migraine with aura compared to migraine without aura, linking the importance of E2 dosage and exposure to trigeminal pain conditions (Gazerani & Cairns, 2020).

We report that acute subcutaneous E2 injections do not exacerbate 5HT-evoked pain behaviors. Indeed it has been reported that, subcutaneous E2 injections do not provide the

physiological levels of E2, whereas the silastic capsule implants (used for steady-state E2 administration) provide a controlled and better E2 release (Aggarwal et al., 2012). We would like to acknowledge that we did not measure serum E2 levels in animals that received acute and steady-state E2 since we are relying on previously published literature (Mannino et al., 2005) to aim for reductionism. Overall, these results are consistent with the 5HT-evoked pain behaviors we reported in intact cycling female rats in Chapters II and III. Literature has consistently used supraphysiological levels of E2 to understand trigeminal pain pathophysiology, whereas our study sheds light on the importance of studying trigeminal pain across physiologically relevant E2 concentrations. Figure 6.1 provides a comparative analysis of 5HT-evoked pain behaviors in presence of different E2 concentrations (endogenous and exogenously administered).

Next, we report that ERα activation enhances the serotonergic potentiation of capsaicin-evoked CGRP release. In support, we reported that E2 pretreatment prior to 5HT treatment enhanced capsaicin-evoked CGRP release and Ca^{2+} influx in cultured trigeminal sensory neurons (Chapter III). Furthermore, blocking the ERα receptor attenuate the enhancement of CGRP release, further strengthening its role in modulating TRPV1 responses. Treatment with agonist/antagonists or ERβ, and membrane bound GPER did not modulate the capsaicin-evoked CGRP release. Thus, E2's effects on trigeminal pain processing appear to be strongly linked to the concentration of E2, the administration time of E2, and the specific ER receptor activated.

Alternatively, the contradicting effects of E2 can be explained by the "mismatch theory" by Welch et al., wherein a loss of balance between the genomic and membrane effects of E2 may underlie the variable effects reported in hormonal modulation of migraine. According to the mismatch theory, a sudden drop in E2 during the cycle may destabilize the gene regulation by E2 thus strengthening the membrane E2 effects eventually leading to hyper excitability and spontaneous CGRP release, thus triggering a migraine attack (Welch et al., 2006).

In addition to E2 fluctuations and exposure, E2 replacement in postmenopausal women can also play a crucial role in trigeminal pain processing depending on the hormone replacement therapy regime. It has been reported that E2 replacement therapy after menopause exacerbates TMD pain (LeResche, 1997). Similarly, in OVX rats, there is an increase in mechanical allodynia in the temporomandibular joint when animals receive daily subcutaneous E2 injections (mimicking hormone replacement therapy). Interestingly, if sub-physiological doses of progesterone are administered along with E2, the allodynia is attenuated (Hornung et al., 2020). While higher E2 in women contribute to the sex differences in trigeminal pain, it's the rapid fluctuations that can either precipitate or ameliorate pain conditions depending on the type of pain disorder (Cairns & Gazerani, 2009).

Another important factor modulating the role of E2+5HT on trigeminal pain processing is the co-expression of ERs and 5HT receptors with TRPV1 ion channel. We report that the nuclear and membrane-bound ERs coexpress with $5HT_{2A}$ and TRPV1 ion channel mRNA in female trigeminal sensory neurons (Chapter V). This provides an anatomical substrate for estrogen to modulate the expression of $5HT_{2A}$ and TRPV1 receptors, act via genomic and membrane-mediated pathways to sensitize the cell, and regulate the gene expression of various pronociceptive genes, ultimately leading to peripheral sensitization. Even though we are measuring mRNA levels and not protein levels, the RNAscope™ technology has high reproducibility and less variability between samples, providing a robust alternative to immunohistochemistry (Shiers et al., 2020).

When we compared the gene expression changes across different steady-state E2 treatment groups, a distinct pattern of differentially expressed genes was reported. In the group that received steady-state supraphysiological dose of E2, we report significantly higher bradykinin 2 receptor (*Bdkrb2*) and prolactin (*Prl*) expression. Interestingly, both bradykinin and

prolactin are pronociceptive and proinflammatory. In fact, ERα activation is reported to enhance bradykinin signaling in cultured female trigeminal sensory neurons (Rowan et al., 2010) and prolactin is reported to play a role in female specific hyperalgesia (Patil et al., 2019). Furthermore, a prolein rich transmembrane protein (*Prrt2*), an important gene in familial migraine, was significantly upregulated in response to supraphysiological E2 treatment (https://www.painresearchforum.org/resources/pain-gene-resource). Also, *Atp1a2*, a sodium potassium ATPase subunit was upregulated in response to steady-state low, high, and supraphysiological E2 treatments. *Atp1a2* also plays an important role in familial migraine (LaPaglia et al., 2018). Lastly, the ionotropic 5HT receptor, $5HT_3$, implicated in serotonergic pain, was also upregulated in supraphysiological E2 group, solidifying the E2-5HT interaction in trigeminal pain disorders (Berman et al., 2006; Domocos et al., 2020).

In contrast, numerous analgesic genes were upregulated in steady-state diestrus E2 group, consistent with attenuation of 5HT-evoked pain behaviors discussed earlier. Notable genes upregulated in trigeminal ganglia post steady-state diestrus E2 were the GABA receptor delta (*Gabrd*), NMDA receptor subunit 3a (*Grin3a*), and Annexin A1 (*Anxa1*), all involved in anti-inflammatory and analgesic roles in trigeminal ganglia pain signaling (Luo et al., 2021; Mohamad et al., 2013; Pei et al., 2011).

Summary – Pro-Nociceptive and Anti-Nociceptive Pathways

This study provides evidence of one interaction that may be underlying the high prevalence of trigeminal ganglia disorders in women. The sensory neurons of the TG innervating the orofacial region can be activated by an incoming noxious stimuli (thermal, mechanical, and chemical). This transient activation is translated to an action potential in response to a persistent stimulus. The resulting signals are then transmitted to higher brain areas (thalamus and cortex) via the medullary dorsal horn, resulting in pain perception and release of proinflammatory

mediators such as CGRP. CGRP can cause vasodilation and recruit immune cells that further exacerbate the pain signaling by creating an inflammatory milieu and releasing numerous inflammatory mediators (5HT). This neuroimmune interaction may be modulated by E2, which results in either an exacerbation or amelioration of the pain processing depending on the concentration and exposure time of E2.

Pro-nociceptive pathway:

We report that, in phases of rapid E2 fluctuations or in presence of a steady-state level of high E2, pain signaling can be exacerbated resulting in peripheral sensitization and neurogenic inflammation. Since estrogen receptors localize on the sensory neurons expression excitatory $5HT_{2A/3A}$ receptors and TRPV1 mRNA, E2 can act via multiple pathways to modulate the overall TG pain signaling. E2 can, (1) enhance 5HT-evoked orofacial nocifensive behaviors and increase levels of 5HT in the TG, resulting in activation of the excitatory $5HT_{2A}$ receptor, (2) enhance release of CGRP from the sensory neurons via ERα, thus exacerbating neurogenic inflammation, (3) increase Ca^{2+} influx and activate downstream kinases that can phosphorylate and sensitize TRPV1, and (4) increase the expression of major nociceptive genes involved in trigeminal ganglia disorders. Ultimately, this results in reduction in activation threshold of TRPV1 and peripheral sensitization, which can exacerbate TG pain signaling (see Figure 6.2).

Anti-nociceptive pathway:

Multiple mechanisms can result in reduction of pain signaling it the TG. In presence of endogenous or exogenous steady-state low level of E2, we report amelioration of the TG pain signaling. Steady state low E2 levels can (1) attenuate 5HT-evoked pain behaviors, and (2) increase the expression of major analgesic genes which may ameliorate trigeminal pain disorders. Additionally, blocking the excitatory $5HT_{2A}$ receptor can also attenuate 5HT-evoked pain behaviors. Furthermore, the nuclear ERα receptor antagonism can reduce the CGRP levels, thus

reducing neurogenic inflammation. Interestingly, E2 can act via membrane-signaling cascades to activate the negative regulator (RSK 1/2/3) of ERK pathway, thus potentially reducing sensitization (see Figure 6.2).

Future Directions Implicated by Our Findings

Future studies warrant a detailed understanding of the (a) molecular mechanisms and (b) peripheral neuroimmune interactions in trigeminal ganglia pain signaling. Multiple molecular mechanisms can regulate TRPV1 modulation and sensitization. Estrogen receptors have a direct and indirect effect on activation of downstream kinases and/ or transcription regulators that can alter trigeminal ganglia transcriptome. Lastly, serotonin, a major inflammatory mediator released by immune cells can also activate downstream secondary messengers that can potentiate TRPV1 activity. Future studies should explore the various kinases activated in response to different ligands/ agonists/ antagonists of the receptors involved (E2, 5HT, and TRPV1). It will be of immense importance to explore any direct physical interaction (heterodimerization / colocalization) between 5HTRs, ERs, and TRP channels. Studies have linked $5HT_7$ to opening of TRPA1 ion channel and $5HT_2$ to TRPV4 ion channels in serotonergic itch (Akiyama et al., 2016; Morita et al., 2015). But, a physical interaction between 5HTRs and TRP channels has not been reported yet. Also, it would be essential to explore the peripheral neuroimmune interaction with respect to role of E2 in modulating immune cell function. E2 can regulate the serotonin synthesizing enzyme and 5HT levels in immune cells. But, it is vital to expand this research to immune cell lines to understand how different concentrations of E2 alter the immune cells function. Considering that sex differences have been reported in immune cell activation, the neuroimmune interaction between resident immune cells in the TGs and sensory neurons will expand our understanding of sexual dimorphism underlying trigeminal pain conditions (Sorge et al., 2015). Lastly, the vast RNA sequencing data obtained from this study should be segregated to

understand: (1) How do different E2 levels modulate kinase expression in the trigeminal ganglia, (2) What GPCRs are differentially expressed in response to E2 treatment, and (3) What genes are differentially expressed in the acute vs steady-state E2 treatment groups.

Clinical Relevance

Despite the sex differences in trigeminal pain conditions, pain studies have majorly been conducted in males. In addition, clinical trials have been male-dominated as well. Our data suggests that it is essential to consider both sexes when designing a behavior study. In addition, sex differences (positive or negative) should be reported when both sexes are present (Mogil, 2012). Furthermore, there is a lot of discrepancy in clinical data when reporting the phase of menstrual cycle and hormone levels in patients. As evident by our data, changes in E2 concentration and exposure time can modulate trigeminal pain processing and should be accurately reported when collecting the background information to treat a patient. The phase of the menstrual cycle and E2 concentration should also be considered when treating trigeminal pain in a clinical setting.

Our data leads us to speculate that when treating trigeminal pain disorders, a clinician should consider: (1) the sex and age of the patient, (2) the menstrual cycle stage and serum E2 levels in case of females, and (3) known sex differences in the effectiveness and adverse effects of the drug (e.g., differences in opioid analgesia). Treatment regimens should also take into account any adverse drug-drug interactions with use of SSRIs, SNRIs, and contraceptive/ hormone pills, specifically because it can have a direct effect on the pain signaling mechanisms. In menopausal women being prescribed hormone replacement, we recommend using a physiologically low dose of E2 or a combined E2 + progesterone regimen to counteract the pronociceptive effects of E2. Lastly, it is essential to consider the history of the patient when deciding on a therapeutic regimen. Important factors to consider are: (1) familial history/ genetic

predisposition to orofacial pain, (2) any previous flares of orofacial pain disorders, and (3) any changes in orofacial pain noted during menarche/ pregnancy. If previous links to menstrual cycle are not known, the patient should be advised to track their orofacial pain over a complete menstrual cycle and advised to follow-up.

Ultimately, it would be helpful if: (1) future clinical trials study and report sex differences in therapeutic response of the drug and (2) a comprehensive database is created that reports sex differences in pain conditions across all developmental stages. In combination, these factors would help in revising the diagnostic criteria and pain management of sexually dimorphic pain conditions.

Table 6.1

Comparison between 5HT-evoked pain behaviors in the TG vs DRG system

Experimental Data	**Hindpaw Study (Dorsal Root Ganglia): Chapter II**	**Orofacial Study (Trigeminal Ganglia): Chapter III**
5HT-evoked pain behaviors	Higher in proestrus and estrus females; not males	Higher in proestrus and estrus females, OVX females; not males
Role of $5HT_{2A}$ receptor	Cycling females and males	Only in proestrus and estrus females; not males
5HT content in interstitial fluid	No differences in females and males	Basal level of 5HT higher in cycling females than males

Figure 6.1

Summary of 5HT-Evoked Orofacial Pain Behaviors in Intact Cycling Female Rats and in OVX Rats Externally Administered with E2

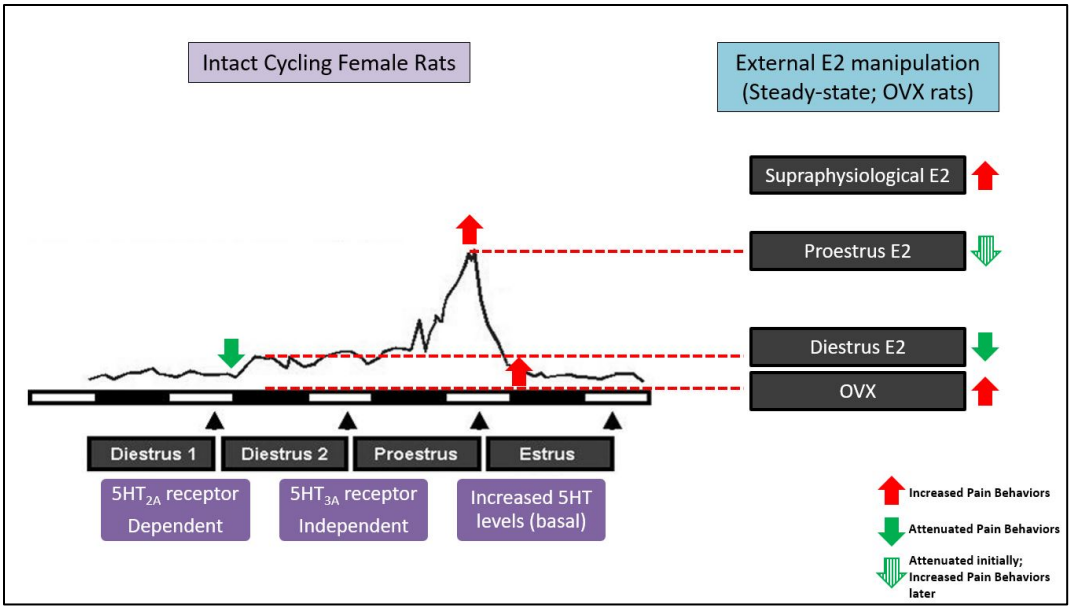
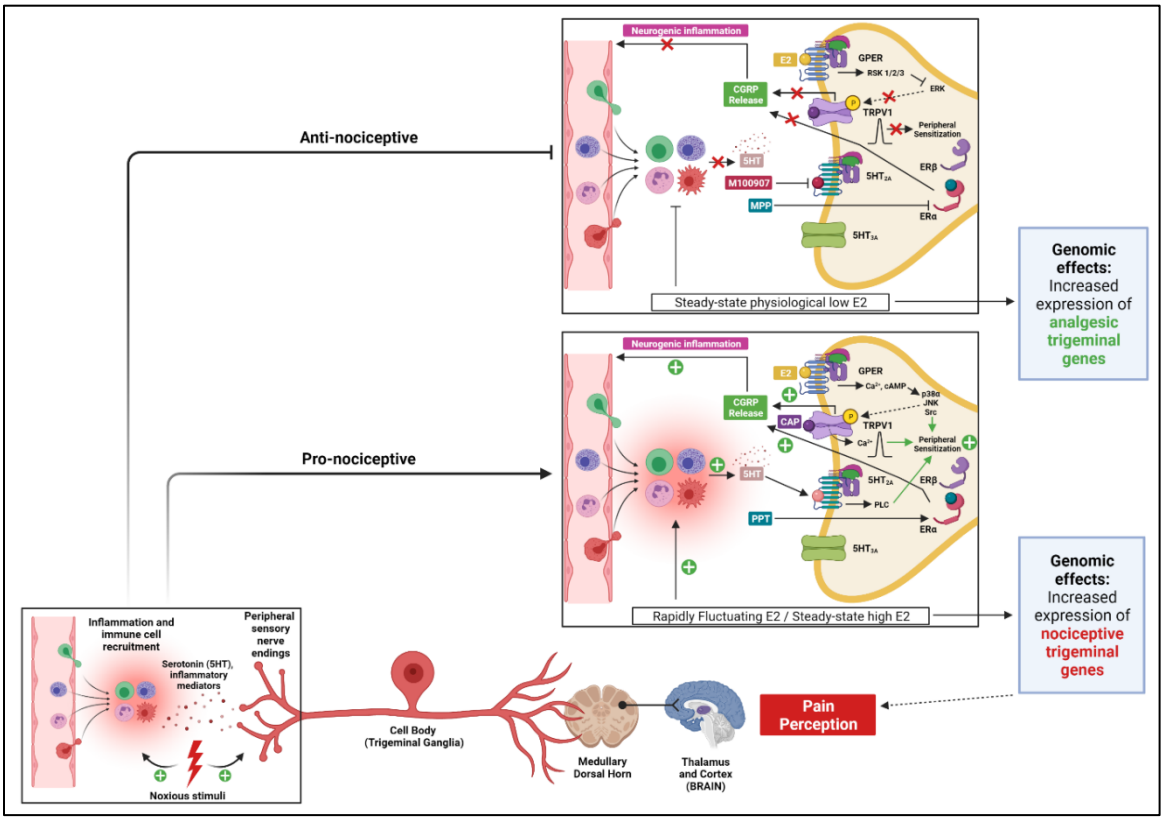


Figure 6.2

Summary of Pro-Nociceptive and Anti-Nociceptive Pathways in the Trigeminal Ganglia



COMPREHENSIVE REFERENCE LIST

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