ROLE OF PROGESTERONE IN SEXUAL RECEPTIVITY AND OROFACIAL PAIN

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DEDICATION

To my husband and best friend, Rick, for his love, encouragement, patience, and support.

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ABSTRACT

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Two understudied disorders that are challenging to treat, sexual dysfunction and orofacial pain, are more prevalent in women and can be impacted by the gonadal hormone's estrogen and progesterone. Estrogen's effects on women's health are well-known, but much less is known about progesterone. The data in this dissertation indicates progesterone may be beneficial in alleviating these two major issues in women. The major drug prescribed for depression increases brain serotonin levels resulting in sexual inhibition, which likely occurs via the serotonin 1A receptor (5-HT_{1A}) as agonists inhibit sexual behavior. Interestingly, progesterone protects against sexual inhibition by an unknown mechanism that may involve the intracellular progesterone receptor (iPR). Here we hypothesized that progesterone's attenuation of 5-HT_{1A} receptor-induced sexual inhibition involves the iPR. Also, progesterone may be beneficial for alleviating temporomandibular joint disorder (TMD) pain, which is 3-4x more common in women. TMD pain dissipates during pregnancy and after menopause but reemerges for some post-

menopausal women prescribed estrogen replacement therapy. Here we hypothesized that therapeutic doses of progesterone in female rats undergoing estrogen replacement would attenuate inflammatory pain behaviors of the temporomandibular joint (TMJ). Ovariectomized, estradiol-primed female rats injected with an iPR antagonist before or after progesterone, then injected with a 5-HT_{1A} receptor agonist had sexual receptivity parameters measured to determine if progesterone's attenuation of 5-HT_{1A} receptor-induced sexual inhibition involves the iPR. To determine progesterone's effect on TMD pain, female rats were tested for basal sensory thresholds at the TMJ then injected with complete Freund's adjuvant (CFA) to trigger inflammation in the TMJ. Mechanical allodynia (developed touch sensitivity) was confirmed then rats were ovariectomized and reassessed for allodynia following various estrogen and progesterone treatment paradigms. This dissertation reports that progesterone acting at the iPR can attenuate 5-HT_{1A} receptor-induced sexual inhibition (Chapter II), progesterone can rapidly attenuate estrogen-evoked TMD pain behaviors (Chapter III), and orofacial sensory neurons contain progesterone-metabolizing enzymes and receptors (Chapter IV). Overall, adjunctive progesterone treatment may provide beneficial gender-based therapies for women facing serotonin-induced sexual dysfunction or post-menopausal women experiencing a return in TMD pain following estrogen treatment.

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LIST OF ABBREVIATIONS

Word	Abbreviation
1. (±)-8-hydroxy 2-(di-n-propylamino) tetralin	8-OH-DPAT
2. 3α-hydroxysteroid oxidoreductase	3α-HSOR
3. 11β-(4-dimethylamino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one	RU-486
4. γ-aminobutyric acid	GABA
5. γ-aminobutyric acid type A receptor	$GABA_A$
6. Analysis of Variance	ANOVA
7. Complete Freund's adjuvant	CFA
8. Cyclic adenosine monophosphate	cAMP
9. Estradiol Benzoate	E2
10. Hormone Replacement Therapy	HRT
11. Intracellular Progesterone Receptor	iPR
12. Membrane-Associated Progesterone Receptors	mPR

13. Ovariectomy ..... OVX

14. Progesterone P4

15. Serotonin 5-HT

16. Selective Serotonin Reuptake Inhibitors ..... SSRI

17. Serotonin Transporter ..... SERT

18. Sigma 1 Receptor Sig-1R

19. Temporomandibular Joint ..... TMJ

20. Temporomandibular Joint Disorder ..... TMD

CHAPTER I

INTRODUCTION

Women's health requires specialized medical attention related to menstruation, pregnancy, and menopause and specialized treatment for other conditions specific to women. These conditions are either directly or indirectly related to the reproductive organs, especially the ovaries that produce the gonadal hormones estrogen and progesterone. Normal fluctuations of ovarian hormones regulate primarily the menstrual cycle, libido, and pregnancy. A hormonal imbalance or ovarian failure to secrete estrogen and progesterone results in menstrual cycle irregularities, menopause, infertility, and sexual dysfunction (Ebrahimi & Akbari Asbagh, 2011; Goswami, Arif, Saxena, & Batra, 2011; Jankowska, 2017; Kingsberg & Woodard, 2015; Prasad et al., 2014). Further, a variety of diseases and disorders are more prevalent in women and may be linked to ovarian hormones. The conditions more prevalent in women include Alzheimer's disease (Alzheimer's Association, 2019), lupus erythematosus (Cooper & Stroehla, 2003), stroke (Chapa, Akintade, Thomas, & Friedmann, 2015), osteoporosis (Wang et al., 2017), rheumatoid arthritis, thyroid diseases (Cooper & Stroehla, 2003), mental or mood disorders (depression and anxiety) (Kessler et al., 2003; McLean, Asnaani, Litz, & Hofmann, 2011; Waraich, Goldner, Somers, & Hsu, 2004), sexual dysfunction

disorders (Laumann, Paik, & Rosen, 1999), and a variety of pain disorders (fibromyalgia, migraine, irritable bowel syndrome, temporomandibular joint disorder; see review in (Manson, 2010). Despite this dimorphism, healthcare professionals continue to use a "one size fits all" treatment and some of the treatments result in side effects that are more severe in women (Goethe, Woolley, Cardoni, Woznicki, & Piez, 2007). Further, some of the treatments appear to be modulated by ovarian hormones (LeResche, Saunders, Von Korff, Barlow, & Dworkin, 1997; Wise, Riley, & Robinson, 2000).

Progesterone, an ovarian hormone mainly known for its role in maintaining pregnancy, has protective effects in preclinical models of Alzheimer's disease (Kaura, Ingram, Gartside, Young, & Judge, 2007), stroke (Cervantes, Gonzalez-Vidal, Ruelas, Escobar, & Morali, 2002), and traumatic brain injury (Pettus, Wright, Stein, & Hoffman, 2005). Progesterone also has protective effects in preclinical models of sexual inhibition (Hassell, Miryala, Hiegel, & Uphouse, 2011; Miryala et al., 2011; Uphouse & Hiegel, 2013) and pain disorders (Liu et al., 2014; Meng, Barton, Goodney, Russell, & Mecum, 2019; Ocvirk, Pearson Murphy, Franklin, & Abbott, 2008). Progesterone is not as heavily studied as estrogen so the mechanism of progesterone's protective effects against sexual inhibition are unknown and less is known about progesterone's role in attenuating pain sensitivity in women. Understanding how progesterone protects against sexual inhibition and pain, two distinct and hard to treat medical conditions prevalent in women, may uncover a

novel gender-based therapeutic regimen that may be more effective than current treatment plans.

Sexual dysfunction is complex due to the influence of hormonal, psychological, physiological, and health-related factors (Laumann, Paik, & Rosen, 1999; Smith, Henderson, Abell, & Bethea, 2004) and can be triggered by the use of therapeutics that increase the amount of the monoamine neurotransmitter serotonin (5-hydroxytryptamine; 5-HT) in some brain areas. 5-HT plays a major role in the pathophysiology of depression and anxiety, and consequently is the major target of pharmacological therapies for these disorders. As depression and anxiety are also 2-3 times more prevalent in women (Lipton et al., 2007; McLean, Asnaani, Litz, & Hofmann, 2011; Steel et al., 2014), women are the major recipients of serotonergic drugs. While effective, serotonergic drugs unfortunately have several negative side effects. One of these side effects, sexual dysfunction, occurs before the onset of clinical efficacy and results in a reduction of the quality of life that often leads women to discontinue their medication (Bondolfi et al., 2006; Bull et al., 2002).

5-HT has both facilitatory and inhibitory effects on sexual behavior, depending on which 5-HT receptor is affected (Maswood, Caldarola-Pastuszka, & Uphouse, 1997; Siddiqui, Niazi, Shaharyar, & Wilson, 2007; Wolf, Caldarola-Pastuszka, DeLashaw, & Uphouse, 1999). Commonly prescribed antidepressants,

such as selective serotonin reuptake inhibitors (SSRIs) (Olfson & Marcus, 2009), increase 5-HT levels in the synapses often leading to sexual dysfunction in up to 70% women (Clayton, Croft, & Handiwala, 2014) are evidence of the inhibitory effects of 5-HT. A commonly prescribed SSRI, fluoxetine, also inhibits sexual behavior in rodents (Adams, Heckard, Hassell, & Uphouse, 2012) which is reversed by a 5-HT_{1A} receptor antagonist (Guptarak, Sarkar, Hiegel, & Uphouse, 2010) indicating that the negative effects of SSRIs on sexual behavior occur when 5HT binds to the $5HT_{1A}$ receptor. In support, the selective 5-HT_{1A} receptor agonist (±) 8-hydroxy 2-(di-n-propylamino) tetraline (8-OH-DPAT) consistently inhibits female rodent sexual behavior (Kishitake & Yamanouchi, 2005; Selvamani, Lincoln, & Uphouse, 2007). Furthermore, both estrogen and progesterone attenuate the effectiveness of 8-OH-DPAT on inhibiting female rodent sexual behavior (Jackson & Uphouse, 1996; Trevino, Wolf, Jackson, Price, & Uphouse, 1999; Truitt et al., 2003). These data indicate ovarian hormones have the capability of reducing $5HT_{1A}$ activity which protects against the negative effects of 5-HT on sexual behavior. The underlying mechanism involves estrogen uncoupling the 5-HT_{1A} receptor from the G protein that inhibits cAMP, ultimately, resulting in an increase in cAMP (Mize & Alper, 2002) and facilitation of sexual behavior (Uphouse, Maswood, & Jackson, 2000). On the other hand, the mechanism whereby progesterone reduces the effectiveness of 8-OH-DPAT on inhibiting sexual behavior is not known, but recent

evidence suggests that the intracellular progesterone receptor (iPR) is involved (Uphouse, 2015).

Progesterone's protective effects are not limited to just female reproductive function. Progesterone reduces anxiety (Engin & Treit, 2007; Gomez, Saldivar-Gonzalez, Delgado, & Rodriguez, 2002; Reddy, O'Malley, & Rogawski, 2005; Ugale et al., 2007), decreases inflammation and edema (Coronel, Labombarda, De Nicola, & Gonzalez, 2014; Coronel et al., 2016; Cutler, Pettus, Hoffman, & Stein, 2005; Cutler et al., 2007; Garay et al., 2012; Grandi et al., 2016; Kasturi & Stein, 2009; Labombarda et al., 2011), improves cognitive function (Cutler, Pettus, Hoffman, & Stein, 2005; Si et al., 2014), and protects against neuronal loss (Barha, Ishrat, Epp, Galea, & Stein, 2011; Cutler, Pettus, Hoffman, & Stein, 2005; Labombarda et al., 2009). However, negative side effects of progesterone are also a concern (Anderson et al., 2012; Chlebowski et al., 2003; Chlebowski et al., 2015). More recently, research indicates that progesterone can reduce both acute and chronic pain sensitivity (Coronel et al., 2011; Coronel, Labombarda, Villar, De Nicola, & Gonzalez, 2011; Coronel, Labombarda, De Nicola, & Gonzalez, 2014; Jarahi, Sheibani, Safakhah, Torkmandi, & Rashidy-Pour, 2014; Liu et al., 2014). Pain can be experienced as acute (lasting minutes to days) or chronic (lasting more than 3 months). Chronic pain is a global health problem because it affects over one quarter of the world's population (IOM, 2011).

Interestingly, women are overrepresented in numerous chronic pain conditions, including fibromyalgia, irritable bowel syndrome, low back pain, migraine, osteoarthritis, rheumatoid arthritis, and temporomandibular joint disorder (TMD) (Wei, Yuan Ong, & Goadsby, 2018). The gender disparity in the predominance of chronic pain disorders reported in women is also reported in pain sensitivity and pain perception. Clinically, women report increased pain sensitivity (Rosseland & Stubhaug, 2004), lower pain threshold, and less tolerance to pain (Fillingim, King, Ribeiro-Dasilva, Rahim-Williams, & Riley, 2009) than men. Women also report that their pain sensitivity or intensity varies across the menstrual cycle (Riley, Robinson, Wise, & Price, 1999; Sherman & LeResche, 2006), with an increase in pain intensity during the luteal phase and menses (Hellstrom & Anderberg, 2003; LeResche, Mancl, Sherman, Gandara, & Dworkin, 2003), thereby, suggesting ovarian hormones play a role in pain modulation. Further, of the chronic pain disorders with a higher prevalence in women, TMD pain appears to be modulated by ovarian hormones because TMD pain varies across the menstrual cycle, decreases after menopause, and returns following hormone replacement therapy (HRT) (LeResche, Mancl, Sherman, Gandara, & Dworkin, 2003).

TMD is one of the most common orofacial pain disorders and is characterized by pain and/or inflammation of the temporomandibular joint, masticatory muscles, and associated tissues. TMD pain affects both men and women, but 75% of cases reported are women (Macfarlane, Glenny, & Worthington, 2001). Ovarian hormones

have been implicated in modulating TMD pain since pain increases after the onset of puberty, peak during reproductive years, dissipates during pregnancy, and after menopause (LeResche, Mancl, Sherman, Gandara, & Dworkin, 2003). Further, some postmenopausal women report a reemergence of TMD pain while undergoing estrogen replacement therapy (LeResche, 1997; Wise, Riley, & Robinson, 2000). Most of the preclinical research on TMD pain focuses predominantly on estrogenic effects while overlooking progesterone's effects.

In preclinical models of TMD pain, estrogen is predominately pronociceptive (Flake, Bonebreak, & Gold, 2005; Kou et al., 2011; Tashiro, Okamoto, & Bereiter, 2009; Wu, Kushwaha, Albert, & Penington, 2002). Estrogen may modulate pain through estrogen-evoked upregulation of inflammatory cytokines (Kou et al., 2011; Puri, Bellinger, & Kramer, 2011; Yun, Chae, & Lee, 2008), modulation of γ-aminobutyric acid type A ($GABA_A$) receptor subunits (Puri, Bellinger, & Kramer, 2011), and upregulation of voltage-gated sodium channels (Bi et al., 2017). On the other hand, progesterone has antinociceptive (Huang et al., 2016; Kim et al., 2012; Liu et al., 2014; Meyer, Patte-Mensah, Taleb, & Mensah-Nyagan, 2010, 2011) and anti-inflammatory properties (Benlloch-Navarro et al., 2019; Lei et al., 2014; Li et al., 2015; Preciado-Martinez et al., 2018), but relatively few studies have investigated the effects of progesterone on TMD pain (Kim et al., 2012; Xue et al., 2017).

TMD pain is relayed from trigeminal nociceptors (pain receptors) innervating the TMJ. Activation of trigeminal nociceptors results in pain during eating, drinking, or talking. The pain signal is relayed to the sensory cell bodies in the trigeminal ganglion, which then sends the signal to the subnucleus caudalis of the trigeminal spinal tract. From here, the signal is relayed to the thalamus (Iwata, Takeda, Oh, & Shinoda, 2017) and then to the somatosensory cortex for pain perception. There is evidence for estrogen targeting the trigeminal ganglia, the trigeminal spinal tract, and the thalamus (Puri, Bellinger, & Kramer, 2011), but to our knowledge no studies have yet reported the effects of progesterone in the trigeminal pain pathway. Furthermore, progesterone can exert effects through intracellular progesterone receptors (iPR), membrane bound progesterone receptors (mPR), or through the progesterone metabolite 5α-pregnan-3α-ol-20-one's (allopregnanolone) activity at $GABA_A$ receptors (see review in (Moussatche & Lyons, 2012; Singh, Su, & Ng, 2013; Thomas & Pang, 2012). However, whether the receptors or the enzymes that metabolize progesterone are expressed in the trigeminal sensory neurons is unknown.

Overall, two major health issues that women experience at higher rates than men that this dissertation targets are sexual dysfunction after SSRIs and TMD pain and our data indicate that progesterone may be protective against both dysfunctions. There is a variety of ways that progesterone can modulate sexual behavior or pain. Progesterone exerts genomic effects through the iPR, which

dimerizes, translocates to the nucleus where it binds the DNA, and acts as a transcription factor regulating target gene expression (Grimm, Hartig, & Edwards, 2016). Progesterone can also inhibit estrogen's ability to modulate gene expression. Progesterone is able to accomplish this when progesterone is bound to the iPR because the iPR will block the estrogen receptor from binding to DNA (Kraus, Weis, & Katzenellenbogen, 1995). Progesterone also has rapid membrane-associated effects through membrane progesterone receptors (mPRs). Five different receptor subtypes have been characterized as G-protein coupled receptors that are coupled to either an inhibitory G protein (mPRα, mPRβ, mPRγ) (Smith et al., 2008; Zhu, Rice, Pang, Pace, & Thomas, 2003) or to a stimulatory G protein (mPRδ, mPRε) (Pang, Dong, & Thomas, 2013).

Progesterone can also have rapid, non-genomic effects through its metabolite 5α-pregnan-3α-ol-20-one (allopregnanolone), which does not bind to the iPR (Gee, Bolger, Brinton, Coirini, & McEwen, 1988) but acts at mPR and $GABA_A$ receptors (Pang, Dong, & Thomas, 2013; Schumacher et al., 2014). Progesterone is first metabolized to 5α-dihydroprogesterone (5α-DHP) by the enzyme 5α-reductase. 5α-DHP is then metabolized by 3α-hydroxysteroid oxidoreductase (3α-HSOR) to allopregnanolone. Allopregnanolone is a potent $GABA_A$ receptor modulator that potentiates the inhibitory function of GABA. Both progesterone and allopregnanolone bind to mPRs, which are expressed within the trigeminal ganglia of rats (Manteniotis et al., 2013), but their role in pain modulation has yet to be

investigated. $GABA_A$ receptor expression has been reported in the trigeminal ganglion, but to our knowledge, there are no reports on whether iPRs, mPRs, or the enzymes that metabolize progesterone to allopregnanolone (5α-reductase or 3α-HSOR) are expressed in the trigeminal ganglion.

Therefore, in the present dissertation, we investigated three overarching hypotheses in female rats: (1) progesterone's protection against sexual inhibition induced by 8-OH-DPAT involves the intracellular progesterone receptor; (2) progesterone attenuates the return of orofacial mechanical allodynia following hormone treatment; (3) the trigeminal ganglia is an anatomical substrate for the actions of progesterone and/or its metabolite allopregnanolone. Results presented herein provide evidence that (1) progesterone's attenuation of sexual inhibition induced by 5-HT_{1A} receptor activation may involve the iPR, (2) both progesterone and 5α-pregnan-3α-ol-20-one (allopregnanolone) rapidly attenuate the return of mechanical allodynia following hormone treatment, and (3) the trigeminal ganglia is a potential anatomical substrate for progesterone. Thus, the use of progesterone as adjunctive therapies may improve the quality of life for women facing serotonin-mediated negative side effects on libido with the use of SSRIs and the return of TMD pain following hormone replacement therapy. Gaining deeper understanding of the mechanisms underlying sexually dimorphic disorders and diseases supports modern gender medicine.

CHAPTER II

RU-486 BLOCKS PROGESTERONE'S ATTENUATION OF 8-OH-DPAT's LORDOSIS-INHIBITING EFFECTS ON FEMALE RAT SEXUAL RECEPTIVITY

A Paper to be Submitted for Publication in Behavioral Brain Research

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Abstract

Gonadal hormones are critical for initiation of female rat sexual behavior. Estradiol is more important, but progesterone allows expression of the entire repertoire of sexual activity. Neurotransmitters also influence the expression of sexual behavior and 5-HT_{1A} receptors have received special attention since 5-HT_{1A} receptor agonists inhibit the behavior. Both estradiol and progesterone reduce the effectiveness of the 5-HT_{1A} receptor agonist, 8-OH-DPAT [(±) -8-hydroxy 2-(di-*n*-propylamino)

tetralin] in inhibiting lordosis behavior. Mechanisms responsible for estrogen's reduction include a decline in 5-HT_{1A} receptor signaling but progesterone's role is not clear. Progesterone also reduces the effects of restraint on sexual behavior and the 5-HT_{1A} receptor has been implicated in this behavioral decline. Since the antiprogestin, RU-486 [(11β-(4-dimethylamino)phenyl-17β-hydroxy-17(1-propynyl)estra-4,9-dien-3-one)], attenuated the effect of progesterone on the response to restraint, the intracellular progesterone receptor was implicated. By extension, the intracellular progesterone receptor may be involved in progesterone's reduction of sexual inhibitory effects of 5-HT_{1A} receptor agonists, but the effect of RU-486 has not been examined. The current project was designed to test this hypothesis. However, it has been reported that RU-486 blocks the serotonin transporter. To rule out the possibility that RU-486 was acting by such a mode, and independent of the intracellular progesterone receptor, RU-486 was administered before or after progesterone treatment. Ovariectomized Fischer rats were primed with 10 μg estradiol benzoate and 500 μg progesterone. RU-486 (5 mg/rat) was administered either 1 hr before progesterone (to block intracellular progesterone receptor-mediated events) or 4 hr after progesterone (when intracellular progesterone receptor-mediated events should already have occurred). Prior to injection with 0-100 μg/kg 8-OH-DPAT, all females showed high lordosis frequency and lordosis quality. However, proceptivity was significantly reduced by RU-486 given before (Chi Square, $p \leq 0.05$), but not after, progesterone. RU-486

treatment before progesterone also enhanced the response to 8-OH-DPAT so that lordosis inhibition was evident at lower doses of the drug (ANOVA, $p \leq 0.05$). Comparable enhancement was not evident when RU-486 was given 4 hr after progesterone. Therefore, the intracellular progesterone receptor may be required for progesterone to reduce the sexual inhibitory effects of 5-HT_{1A} receptor activation.

Keywords: progesterone, RU-486, proceptivity, lordosis, sexual behavior, 5-HT_{1A} receptors

1. Introduction

Female rat sexual behavior, which requires the ovarian hormones, estrogen and progesterone, includes proceptive and receptive behaviors (Bergheim, Chu, & Agmo, 2015; Jones, Gardner, & Pfaus, 2015; Sanathara, Moreas, Mahavongtrakul, & Sinchak, 2014). Proceptive behaviors, such as hopping and darting, are solicitous behaviors displayed by the female toward a male to entice him to mate with her (Bergheim, Chu, & Agmo, 2015; Micevych, Soma, & Sinchak, 2008). Lordosis, the dorsoventral arching of the back, is a receptive behavior displayed by the female in response to tactile stimulation by the male (Bergheim, Chu, & Agmo, 2015; Micevych, Soma, & Sinchak, 2008). Estrogen is required for the onset of sexual behaviors and the female's receptivity, but it is thought that progesterone (P4) is required for proceptive behaviors and for enhancement of lordosis (Jones, Gardner,

& Pfaus, 2015). Progesterone-facilitated sexual behavior requires the intracellular progesterone receptor (iPR). When antisense oligonucleotides to the iPR were infused into the 3rd ventricle or the ventromedial nucleus of the hypothalamus of rodents, a decrease in progesterone-facilitated sexual behavior was observed (Mani et al., 1994; Ogawa, Olazabal, Parhar, & Pfaff, 1994). Similarly, rats treated with RU-486 (11β-(4-dimethylamino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one), an antagonist to the iPR (Shatnawi, Tran, & Ratnam, 2007), or iPR knockout mice each had a significant decrease in P4-facilitated sexual behavior (Mani et al., 1996; Micevych, Soma, & Sinchak, 2008). Progesterone interacts with the iPR, which acting as a transcription factor, results, in regulation of gene expression, or progesterone can have rapid non-genomic actions through membrane-associated progesterone receptors (mPRs) (Grimm, Hartig, & Edwards, 2016; Singh, Su, & Ng, 2013). Recently, progesterone interaction with the mPRS has also been shown to facilitate female rat sexual behavior (Frye & Vongher, 1999; Frye, Walf, Kohtz, & Zhu, 2013, 2014) and antisense oligonucleotides to mPRs reduced progesterone's effect on sexual behavior (Frye, Walf, Kohtz, & Zhu, 2013, 2014).

Although ovarian hormones are required for the onset of female rat sexual behaviors, the expression of these behaviors can be influenced by neurotransmitters, such as serotonin (5-HT) (Frye & Paris, 2009; Graham & Pfaus, 2010; Wada, Yamada, & Yamanouchi, 2008). Depending on which of the 5-HT receptors are affected, 5-HT receptor agonists or antagonists can lead to either

inhibition or facilitation of sexual behavior (Maswood, Caldarola-Pastuszka, & Uphouse, 1997; Siddiqui, Niazi, Shaharyar, & Wilson, 2007; Uphouse et al., 2003; Uphouse & Wolf, 2004; Wolf, Caldarola-Pastuszka, DeLashaw, & Uphouse, 1999). The role of 5-HT_{1A} receptors in female rodent sexual behavior is most clearly defined. The 5-HT_{1A} receptor agonist, 8-OH-DPAT ((±)-8-hydroxy 2-(di-n-propylamino) tetralin), consistently inhibits sexual behavior (Kishitake & Yamanouchi, 2005; Selvamani, Lincoln, & Uphouse, 2007; Uphouse & Wolf, 2004) and both estrogen and progesterone reduce the effectiveness of 8-OH-DPAT (Jackson & Uphouse, 1996; Trevino, Wolf, Jackson, Price, & Uphouse, 1999; Truitt et al., 2003). Estrogen reduces 8-OH-DPAT's effectiveness by reducing 5-HT_{1A} receptor signaling due to uncoupling between the receptor and its Gi protein (Mize & Alper, 2002); but the mechanism by which progesterone reduces 8-OH-DPAT's effectiveness is not known.

In an earlier study, RU-486 was used to determine if the iPR was involved in progesterone's attenuation of the negative effects of a mild restraint on female rat sexual behavior (Hassell, Miryala, Hiegel, & Uphouse, 2011). In this paradigm, RU-486 blocked the attenuating effects of progesterone on restraint. In order to eliminate the possibility that progesterone had been converted to its metabolites, the authors investigated inhibitors of progesterone metabolism and these did not alter the attenuating effects of progesterone on the response to restraint (Miryala et al., 2011). Moreover, 8-OH-DPAT amplified the effects of the restraint and

progesterone also attenuated the effects of 8-OH-DPAT (Truitt et al., 2003). Thus, we hypothesized that the iPR might be involved in the attenuation of 8-OH-DPAT's inhibition of sexual behavior.

In the following studies, the effects of RU-486 on 8-OH-DPAT's inhibition of sexual behavior were examined in rats after hormonal priming with estradiol benzoate and progesterone. Since recently, it has been suggested that RU-486 can block the serotonin transporter (SERT) (Li, Shan, Li, Wei, & Li, 2014) and since blocking SERT would increase extracellular serotonin and hence activation of 5-HT_{1A} receptors (Dankoski, Carroll, & Wightman, 2016; Pineyro & Blier, 1999), two different paradigms were investigated. RU-486 was administered either 1 hr before the progesterone injection or 4 hr after the progesterone injection. If RU-486 reduced progesterone's attenuation of effects of 8-OH-DPAT by blocking the iPR, the antiprogestin should be effective when administered before but not after progesterone. Alternatively, if RU-486 were effective when given after progesterone, another mechanism such as the SERT would be implicated. It was hypothesized that RU-486 would be more effective when administered before progesterone.

2. Methods and Materials

2.1 Materials

Estradiol benzoate (E2), progesterone (P4), the progesterone receptor antagonist [RU-486 (11β-(4-dimethylamino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one)], the 5-HT_{1A} receptor agonist [8-OH-DPAT (±)-8-hydroxy 2-(di-n-propylamino) tetralin], dimethyl sulfoxide (DMSO), and sesame seed oil were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Propylene glycol was obtained from Eastman Kodak Company (Rochester, NY). Isoflurane (AErrane®) was purchased from Butler Schein Animal Health (Dublin, OH).

E2 and P4 were dissolved in sesame seed oil and administered subcutaneously (sc). RU-486 (5 mg/rat) was dissolved in 15% DMSO with propylene glycol and administered sc in a volume of 0.4 ml. 8-OH-DPAT was dissolved in saline and administered intraperitoneally (ip) at a value of 1 ml/kg.

2.2 Animals, housing and treatment procedures

Adult Fischer female rats were purchased from Charles River Laboratories (Wilmington, MA) and were housed 2 to 3 per polycarbonate shoebox cage in a colony room with lights off from 12 noon to 12 midnight. Food and water were available *ad libitum*. The colony room was maintained at 25 °C with 55% humidity.

After at least a two-week acclimation to the facility, females (at least 150 g), were anesthetized with AErrane® and ovariectomized as previously described (White & Uphouse, 2004). Approximately two weeks after surgery, rats were used in the experiments.

For the first paradigm, the rats were hormonally primed with 10 μg EB, followed 47 h later with RU-486 (5 mg per rat) or vehicle (15% DMSO + propylene glycol) and one hour later were injected with P4 (500 μg/rat). Testing occurred 4 hr after progesterone. For the second paradigm, RU-486 was administered 4 hr after progesterone and rats were tested one hr later.

2.3 Evaluation of sexual behavior

Sexual behavioral testing was monitored as previously described (White and Uphouse, 2004). For each mount by the male, the presence or absence of a lordosis reflex was recorded. L/M ratios (number of lordosis responses to male's mounts divided by the number of mounts by the male), lordosis quality (magnitude of the female's back arching, scored on a scale of 1 to 4), resistance, and proceptivity were recorded. The existence of hopping and darting was recorded as proceptive behavior, whereas, rolling over, kicking, boxing, or running away from the male were recorded as resistive behaviors.

2.4 Testing for sexual behavior

For the pretest, the female rat was placed into the home cage of a sexually experienced male Sprague Dawley and behavior was monitored for 10 minutes or 10 mounts. The female was removed, injected with the saline vehicle or 50, 75, or 100 μg/kg body weight of 8-OH-DPAT, and returned to the male's cage for an additional 15 minutes of behavioral testing.

2.5 Statistical procedures

When effects of RU-486 were examined, data for the pretest before 8-OH-DPAT were examined. Data were examined by analysis of variance (ANOVA) or Chi-Square as described below. All animals were included in the pretest analyses, but when effects of RU-486 on the response to 8-OH-DPAT were examined, only animals with an initial pretest L/M ratio ≥ 0.6 were included. For these animals, L/M ratios and lordosis quality before and after injection of 8-OH-DPAT or the saline vehicle were evaluated by a three-way repeated measures ANOVA with time relative to injection of 8-OH-DPAT as the repeated factor and treatment with RU-486 or vehicle, time of RU-486 or vehicle, and dose of 8-OH-DPAT as independent factors. Post hoc comparisons were made with Tukey's test. Proceptivity and resistance were compared by Chi square test procedures. Estimates of IC_{50} values for 8-OH-DPAT's effect on L/M ratios were made with linear regression procedures

with Y = 0.5 L/M ratio as 50% inhibition. Data were analyzed with SPSS v. 19 and the statistical reference was (Zar, 2018).

3. Results

3.1 Effects of RU-486 on pretest behavior

Since prior investigators have reported L/M reducing effects of RU-486 (11β-(4-dimethylamino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one) in E2 and progesterone-treated rats (Dominguez-Ordonez et al., 2018; Hassell, Miryala, Hiegel, & Uphouse, 2011; Jones, Gardner, & Pfaus, 2015), behavior in the pretest was examined before analyzing effects of 8-OH-DPAT. A total of 136 rats were used. Data are summarized in Table 1. The majority of rats had pretest lordosis to mount (L/M) ratios greater than 0.6. However, 5 rats (1 vehicle in the afternoon, 1 RU-486 in the morning, and 3 RU-486 in the afternoon) had L/M ratios < 0.60 (from 0.40 to 0.56) and, as a consequence, there was a small but significant effect of RU-486 (ANOVA $F_{1,132} = 10.417$; $p \leq 0.002$) on pretest L/M ratios which was greater when RU-486 preceded P4 by 1 hr. When RU-486 was given after P4, behavior did not differ from the vehicle ($p > 0.05$). Nevertheless, the absolute difference among groups was small (see Table 1). These findings are consistent with prior reports (Hassell, Miryala, Hiegel, & Uphouse, 2011; Miryala et al., 2011) that 10 μg E2 given to Fischer rats produces a relatively high L/M ratio even in the absence of progesterone. Consequently, even if progesterone's action is blocked with RU-486,

the effect on L/M ratios is small. This contrasts with Sprague-Dawley rats where progesterone addition to E2 is required for high receptivity (Beyer, Gonzalez-Flores, & Gonzalez-Mariscal, 1995; Mani et al., 1994).

Not surprisingly, there was not a significant effect of treatment on the quality of lordosis in the pretest (ANOVA $F_{1, 132} = 2.873$, $p \leq 0.092$). However, RU-486 did significantly reduce proceptivity and increase resistance. Although progesterone is not required for lordosis (Sanathara, Moreas, Mahavongtrakul, & Sinchak, 2014), it is essential for proceptivity and, as anticipated, proceptivity in the pretest was reduced when RU-486 was given an hour before progesterone (Chi Square $df = 1$, $p \leq 0.039$). However, RU-486 did not affect proceptivity when given 4 hours after progesterone (Chi square $df = 1$, $p > 0.05$). These findings are consistent with prior reports (Hassell, Miryala, Hiegel, & Uphouse, 2011; Jones, Gardner, & Pfaus, 2015) that RU-486 given before progesterone blocks proceptivity.

The effects of RU-486 on resistance paralleled the antiprogestin's effect on proceptivity. Resistance was significantly increased when RU-486 was given an hour before progesterone (Chi Square $df = 1$, $p \leq 0.001$) but not when given 4 hours after progesterone (Chi Square $df = 1$, $p > 0.05$).

3.2 Effects of RU-486 on the response to 8-OH-DPAT

Immediately after the pretest, rats with L/M ratios ≥ 0.6 were injected with 8-OH-DPAT [(±) -8-hydroxy 2-(di-*n*-propylamino) tetralin]. Consistent with prior

reports (Kishitake & Yamanouchi, 2005; Selvamani, Lincoln, & Uphouse, 2007), 8-OH-DPAT significantly inhibited lordosis behavior. There was a significant main effect of dose of 8-OH-DPAT (ANOVA $F_{3, 101} = 18.752$; $p \leq 0.001$) as well as a significant time after injection by dose of 8-OH-DPAT interaction (ANOVA $F_{3, 101} = 28.393$; $p \leq 0.001$) (see Figure 2). RU-486 enhanced the effects of 8-OH-DPAT (ANOVA $F_{1, 101} = 12.154$; $p \leq 0.001$) and this was most evident when RU-486 was given one hour before progesterone. Relative to their pretest, in rats treated with RU-486 1 hr before progesterone, L/M ratios were significantly reduced at 50, 75 and 100 μg 8-OH-DPAT (all Tukey's $q_{101,16} > 5.022$, $p \leq 0.05$) while a decline in the vehicle-treated rats was evident only with 100 μg 8-OH-DPAT (Tukey $q_{101, 16} = 8.95$, $p \leq 0.05$). Consequently, L/M ratios of RU-486-treated rats were significantly different from vehicle-treated rats at 50 μg 8-OH-DPAT ($q_{101, 16} = 5.12$, $p \leq 0.05$). Although 8-OH-DPAT reduced L/M ratios in rats given RU-486 4 hr after progesterone, there were no significant differences between RU-486 and vehicle-treated rats. Since 8-OH-DPAT reduced L/M ratios in all groups, there was not a significant RU-486 time by dose of 8-OH-DPAT interaction. Instead, RU-486 shifted the dose response for 8-OH-DPAT and reduced the estimated IC_{50} for 8-OH-DPAT (IC_{50} respectively, vehicle 1 hr = 111.4, RU-486 1 hr = 60.33, vehicle 4 hr = 100.2, RU-486 4 hr = 84.3 μg 8-OH-DPAT)

There were only minor effects of either RU-486 or 8-OH-DPAT on lordosis quality. Nevertheless, there was an overall main effect of vehicle vs RU-486 ($F_{1,93} =$

5.371, $p \leq 0.023$) as well as a significant effect of the time of RU-486 treatment (ANOVA $F_{1, 93} = 8.128$; $p \leq 0.005$; see figure 3). A significant effect of dose of 8-OH-DPAT by time (before or after 8-OH-DPAT) interaction (ANOVA $F_{3, 93} = 2.902$; $p \leq 0.039$) reflected a small effect of 8-OH-DPAT on lordosis quality. Nevertheless, all lordosis quality scores were ≥ 2.30.

Effect of 8-OH-DPAT on proceptivity is shown in Table 1. Consistent with effects of RU-486 on proceptivity in the pretest, proceptivity after 8-OH-DPAT remained different between vehicle and the 1 hr RU-486-treated rats (Chi Square, 1*df* = 5.796, $p \leq 0.036$) but not the 4 hr RU-486-treated rats (Chi square, 1 *df* = 0.217, $p \geq 0.05$). Thus, these effects could be attributed primarily to RU-486 and not to 8-OH-DPAT. 8-OH-DPAT did not further significantly alter proceptivity in either vehicle or RU-486-treated rats ($p > 0.05$).

Effect of 8-OH-DPAT on resistance is shown in Table 1. While RU-486 given 1 hr before progesterone had a significant effect on resistance before 8-OH-DPAT (Chi Square, 1 *df* = 8.869, $p \leq 0.003$), there was no difference between vehicle or 1 hr RU-treated rats after 8-OH-DPAT (Chi Square, 1 *df* = 0.266, $p > 0.05$). Treatment with RU-486 4 hr after progesterone also had no effect on the resistance response to 8-OH-DPAT (Chi Square, 1 *df* = 0.215, $p > 0.05$) and there was no difference between vehicle or 4 hr RU-486-treated rats (Chi Square, 1 *df* = 0.042, $p > 0.05$).

4. Discussion

The objective of this experiment was to test the hypothesis that progesterone's reduction of the effect of 8-OH-DPAT [(±) -8-hydroxy 2-(di-*n*-propylamino) tetralin] on female rat sexual behavior involved the iPR. If so, it was hypothesized that the antiprogestin, RU-486 (11β-(4-dimethylamino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one), by acting as an antagonist for the iPR, would attenuate the effect of progesterone. However, since it has been suggested that RU-486 might block the serotonin transporter (SERT) (Li, Shan, Li, Wei, & Li, 2014), this possibility was also taken into account. Serotonin reuptake inhibitors (SSRIs), which result in an increase of extracellular serotonin (5-HT) (Pineyro & Blier, 1999) can increase the amount of 5-HT available for 5-HT_{1A} receptor activation. This increased 5-HT_{1A} receptor activation can then inhibit sexual behavior (Guptarak, Sarkar, Hiegel, & Uphouse, 2010). Since blocking SERT, alone, can inhibit female rat sexual behavior, it was essential to differentiate the antiprogestin effects of RU-486 from its potential SERT actions. To accomplish this differentiation, we administered RU-486 before or after progesterone treatment. If, as hypothesized, the intracellular progesterone receptor (iPR) were involved in progesterone's attenuation of the effects of 8-OH-DPAT, then RU-486 should block the effect of progesterone when given before, but not after, progesterone treatment.

While progesterone is not required for the onset of expression of sexual behavior (Micevych, Soma, & Sinchak, 2008), progesterone does facilitate estradiol-inducted lordosis behavior and is thought to be required (by acting through the iPR) for proceptive behaviors. Because RU-486 can reduce progesterone-facilitated lordosis behavior, it was essential to utilize a rat model (such as the Fischer strain; (Hassell, Miryala, Hiegel, & Uphouse, 2011; Miryala et al., 2011) where high levels of lordosis behavior were present in the absence of progesterone. That RU-486 did not reduce estradiol-primed lordosis behavior, but did reduce proceptivity and increase resistance, is consistent with the importance of progesterone for female solicitious behaviors. These results are consistent with previous studies where only a slight decrease in lordosis to mount ratio was observed following administration of RU-486 (Hassell, Miryala, Hiegel, & Uphouse, 2011; Jones, Gardner, & Pfaus, 2015; Miryala et al., 2011), indicating intracellular progesterone receptors are not required for estradiol-induced lordosis but they may be required for progesterone's facilitation of estradiol -induced lordosis. Moreover, the greater effect of RU-486 on both proceptivity and resistance when given before rather than after progesterone is consistent with RU-486's antagonism of iPRs. The low proceptivity and high resistance after RU-486, in the absence of 8-OH-DPAT, prevented assessment of the antiprogestin's effect on these behaviors after the 5-HT_{1A} receptor agonist.

In contrast to these solicitous behaviors, there was only a small decrease in the L/M ratio when RU-486 was administered prior to 8-OH-DPAT. Therefore, it

was possible to examine the effects of RU-486 on 8-OH-DPAT's inhibition of lordosis behavior. Consistent with earlier studies (Kishitake & Yamanouchi, 2005; Selvamani, Lincoln, & Uphouse, 2007), 8-OH-DPAT reduced lordosis behavior and progesterone attenuated the effects of 8-OH-DPAT (Truitt et al., 2003). RU-486 attenuated the effect of progesterone but only when RU-486 was administered before progesterone. This finding is consistent with our hypothesis that progesterone reduces the effect of the 5-HT_{1A} receptor agonist in part by blocking the iPR.

Quality of lordosis, the degree of arching of the back, was not affected by RU-486 administration before or after progesterone indicating that quality of lordosis is not dependent upon progesterone interacting with the intracellular progesterone receptor (Micevych & Meisel, 2017). Similarly, consistent with earlier studies, 8-OH-DPAT had only minor effects on lordosis quality and this was not impacted by RU-486.

RU-486 is a selective progesterone receptor modulator (SPRM) that binds either isoform of the iPR blocking progesterone's actions at the iPR (Baulieu, 1991). While RU-486 is best known to be used for medical termination of a pregnancy, it has also been used to induce labor in full-term pregnant women (Baev, Rumyantseva, Tysyachnyu, Kozlova, & Sukhikh, 2017). SPRMs have recently been used as a therapeutic treatment for endometrial bleeding (Fu et al., 2017), uterine

fibroids (Arora, Chawla, Kochar, & Sharma, 2017; Liu et al., 2017; Murji, Whitaker, Chow, & Sobel, 2017), and leiomyomata (Jain, 2018; Mukherjee & Chakraborty, 2011; Murphy, Morales, Kettel, & Yen, 1995; Yerushalmi et al., 2014). As previously mentioned, RU-486 has been suggested to have blocking actions on SERT (Li, Shan, Li, Wei, & Li, 2014) and this putative mechanism might contribute to the ability of RU-486, administered prior to chronic stress, to downregulate the stress-induced upregulation of SERT (Zhang et al., 2012). Various stressors (forced swim, tail pinch, immobilization, cold, and predator stress) have been shown to increase extracellular 5-HT levels in rats (Kirby, Chou-Green, Davis, & Lucki, 1997) and mice (Beekman, Flachskamm, & Linthorst, 2005; Fujino et al., 2002). Increased levels of extracellular 5-HT from stressors or drugs that alter the serotonergic system inhibit lordosis presumably through the 5-HT_{1A} receptor (Guptarak, Sarkar, Hiegel, & Uphouse, 2010). Therefore, if RU-486 has actions that increase extracellular serotonin, it would be expected to be effective at inhibiting lordosis

Progesterone reduces the lordosis-inhibiting effects of SSRI's (Frye, Bayon, Pursnani, & Purdy, 1998; Frye, Petralia, Rhodes, & Stein, 2003; Frye, 2007; Frye & Rhodes, 2010; Guptarak, Sarkar, Hiegel, & Uphouse, 2010), stressors (Frye & Walf, 2002; Khisti, Chopde, & Jain, 2000; Truitt et al., 2003; Uphouse, Adams, Miryala, Hassell, & Hiegel, 2013; Uphouse & Hiegel, 2013, 2014), and 5-HT_{1A} agonists (Guptarak, Sarkar, Hiegel, & Uphouse, 2010; Truitt et al., 2003) and this appears to result from the hormone's action at iPRs. Nevertheless, while progesterone has

genomic actions through the iPR, which acts as a transcription factor to regulate gene expression (Abdel-Hafiz & Horwitz, 2014; McKenna, Lanz, & O'Malley, 1999; Scarpin, Graham, Mote, & Clarke, 2009), progesterone also has rapid, membrane-associated effects that activate intracellular signaling cascades instead of directly interacting with the iPR (Falkenstein, Tillmann, Christ, Feuring, & Wehling, 2000; Losel & Wehling, 2003). Rapid, membrane-associated protective effects of progesterone against inhibition of lordosis have been attributed to membrane progesterone receptors (mPRs) (Frye, Walf, Kohtz, & Zhu, 2013, 2014). The iPR is required for G-protein coupled estrogen receptor 1 (GPER-1) induction of lordosis (Dominguez-Ordonez et al., 2018) and attenuation of the negative effects of restraint stress on lordosis (Hassell, Miryala, Hiegel, & Uphouse, 2011; Uphouse, Adams, Miryala, Hassell, & Hiegel, 2013; Uphouse & Hiegel, 2013, 2014). Progesterone can also be metabolized to allopregnanolone, which has membrane-associated effects, through actions at mPRs and $GABA_A$ receptors, and does not bind to the iPR. Allopregnanolone, in the ventral tegmental area is important for the lordosis behaviors (Frye, 2001; Frye & Vongher, 2001; Frye & Rhodes, 2006; Petralia, Walf, & Frye, 2006). Allopregnanolone has been shown to attenuate the lordosis-inhibiting effects of restraint but the attenuation seemed to require the iPR (Hassell, Miryala, Hiegel, & Uphouse, 2011; Miryala et al., 2011; Uphouse, Adams, Miryala, Hassell, & Hiegel, 2013; Uphouse & Hiegel, 2014). Here, we show that RU-486 prevented progesterone from attenuating the lordosis-inhibiting effects of 5-

HT_{1A} activation. This supports a potential role for the iPR in progesterone's protection from 5-HT_{1A} receptor activation. However, since RU-486 has glucocorticoid activity, further studies with an iPR antagonist, such as CDB-4214, that has no glucocorticoid activity are warranted.

In summary, RU-486 attenuated the ability of progesterone to reduce the lordosis-inhibiting effects of 8-OH-DPAT in estradiol-primed female rats. This was more evident when RU-496 was administered before, and not after, progesterone injection. Consequently, the iPR may be involved in progesterone's protection from the negative effects of 8-OH-DPAT has on lordosis behavior.

5. Conflict of Interest

The authors declare no conflicts of interest.

6. Author Contributions

RSH contributed to experimental design, conducting experiments, data analysis and interpretation, and preparation of the manuscript. LU contributed to experimental design, data analysis and interpretation, and preparation of the manuscript.

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Table 1.

Effects of RU-486 and response to 8-OH-DPAT

	Vehicle 1 hr (Mean ± S.E.)	RU-486 1 hr (Mean ± S.E.)	Vehicle 4 hr (Mean ± S.E.)	RU-486 4hr (Mean ± S.E.)
Pretest Behavior before 8-OH-DPAT (all animals)[A]				
Initial pretest n	28	41	33	34
Pretest mean (S.E.) L/M ratio	0.99 (0.024)	0.89 (0.020)	0.97 (0.022)	0.93 (0.022)
Pretest mean (S.E.) Lordosis quality	2.95 (0.034)	2.85 (0.028)	2.95 (0.031)	2.94 (0.031)
Number (%) proceptive in pretest	23 (82.1%)	24 (58.5%)*	23 (69.7%)	25 (73.5%)
Number (%) resistant in pretest	10 (35.7%)	33 (80.5%)*	13 (39.4%)	15 (44.1%)
Number (%) with pretest $\geq$ 0.60	28	40	32	31
Number missing mounts after treatment with DPAT	2	7	4	1
Behavior of rats after treatment with 8-OH-DPAT (rats with pretest L/M $\geq$ 0.60)[B]				
Final n for DPAT treatment analysis	26	34	28	30
Number (%) proceptive after DPAT	6 (23.1%)	1 (2.94%)	6 (21.4%)	8 (26.6%)
Number (%) resistant after DPAT	22 (84.6%)	27 (79.4%)	18 (64.3%)	21 (70%)

[A] Behavior of all animals before treatment with DPAT.

[B] Only rats with pretest L/M $\geq$ 0.60 were included in DPAT procedure; pretest behavior of this subset of rats is included in describing data before DPAT treatment.

*significantly different from vehicle of same time

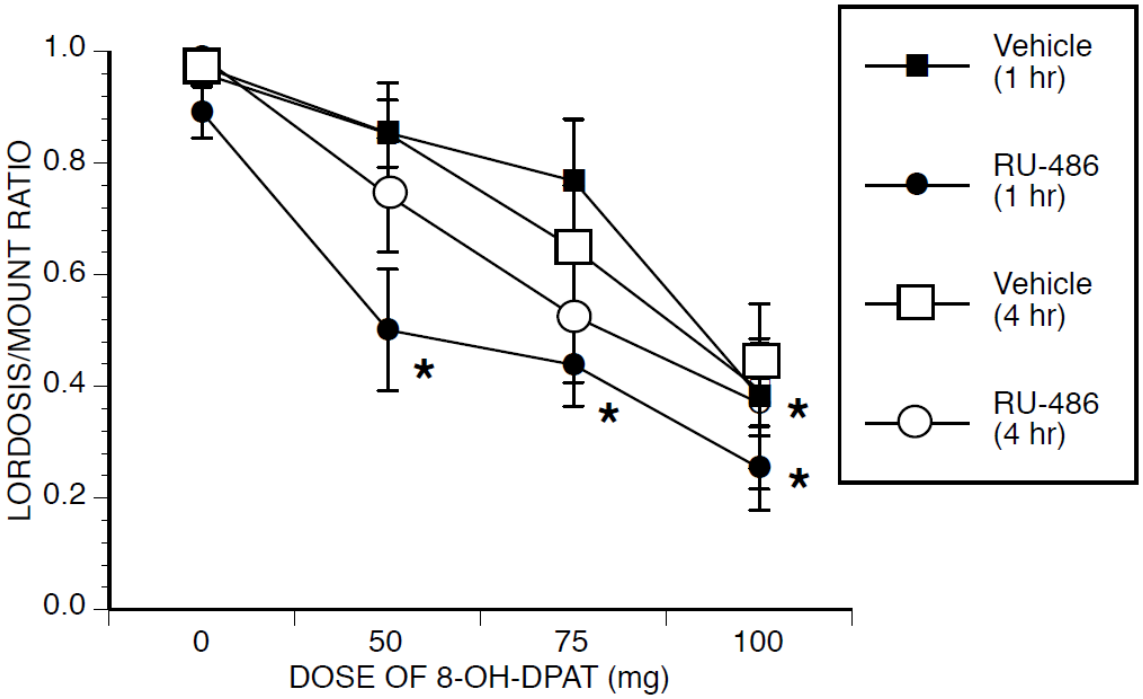

Figure 1. Effects of RU-486 on L/M ratios following pretest evaluation. After pretest, rats that had ≥ 0.6 L/M ratio were injected i.p. with saline or 50, 75, or 10 ug/kg of 8-OH-DPAT. Data are the mean ± S.E. N's for rats injected with vehicle (1 hr), RU-486 (1 hr), vehicle (4 hr), or RU-486 (4 hr) were 26, 34, 28, 30 respectively. indicates a significant difference relative to vehicle of the same time and dose of 8-OH-DPAT.

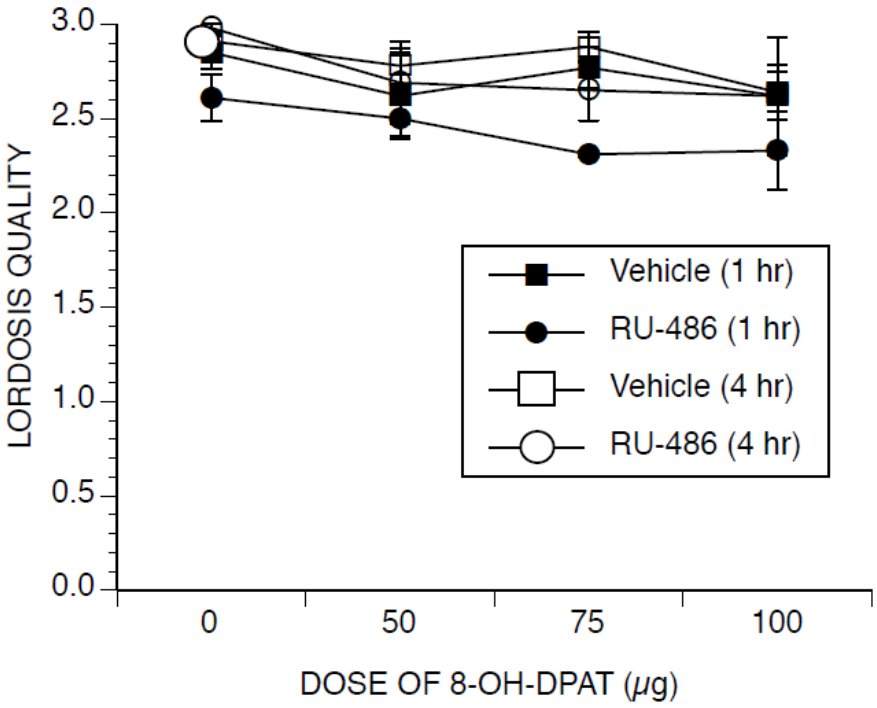

Figure 2. Effects of RU-486 on lordosis quality following pretest evaluation. After pretest, rats that had ≥ 0.6 L/M ratio were injected i.p. with saline or 50, 75, or 10 ug/kg of 8-OH-DPAT. Data are the mean ± S.E. N's for rats injected with vehicle (1 hr), RU-486 (1 hr), vehicle (4 hr), or RU-486 (4 hr) were 26, 34, 28, 30 respectively.

CHAPTER III

PROGESTERONE AND ALLOPREGNANOLONE RAPIDLY ATTENUATE ESTROGEN-ASSOCIATED MECHANICAL ALLODYNIA IN RATS WITH PERSISTENT TEMPOROMANDIBULAR JOINT INFLAMMATION

A Paper Under Revisions for the Frontiers of Integrative Neuroscience Journal

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Abstract

Temporomandibular joint disorder (TMD) is associated with pain in the joint (TMJ) and muscles involved in mastication. TMD pain dissipates following menopause but returns in some women undergoing estrogen replacement therapy. Progesterone

has both anti-inflammatory and antinociceptive properties, while estrogen appears to be primarily pronociceptive. Allopregnanolone, a progesterone metabolite and positive allosteric modulator of the $GABA_A$ receptor, also has antinociceptive properties. While progesterone and allopregnanolone are antinociceptive, their effect on TMD pain has not been determined. We hypothesized that removing the source of endogenous ovarian hormones would reduce inflammatory allodynia in the TMJ of rats and both progesterone and allopregnanolone would attenuate the estrogen-provoked return of allodynia. Baseline mechanical sensitivity was measured in female Sprague Dawley rats (150-175 g) using the von Frey filament method followed by a unilateral injection of complete Freund's adjuvant (CFA; 30 µl, 1:1 in saline) into the TMJ. Mechanical allodynia was confirmed 24 hours later; then rats were ovariectomized or received sham surgery. Two weeks later, allodynia was reassessed and rats received one of the following subcutaneous hormone treatments over 5 days: daily estradiol benzoate (E2; 50 µg/kg), daily E2 and progesterone (P4; 16 mg, µg, or ng/kg), E2 daily and interrupted P4 given every other day (on days 1, 3, and 5), daily P4, or daily vehicle control. A separate group of animals was tested using the same paradigm but received allopregnanolone (0.16 mg/kg) instead of P4. Allodynia was reassessed one hour following injections on days 1, 3, and 5. We report that CFA-evoked mechanical allodynia was attenuated following ovariectomy while sham animals remained allodynic. Daily E2 treatment triggered the return of allodynia, which was attenuated when P4 was also

administered either daily or every other day. This attenuation occurred rapidly within one hour of administration. Allopregnanolone treatment, whether daily or every other day, also attenuated estrogen-exacerbated allodynia within one hour of treatment, but only on the first treatment day. These data indicate that when gonadal hormone levels have diminished, treatment with a low dose of progesterone may be effective at rapidly reducing the estrogen-evoked recurrence of inflammatory mechanical allodynia in the TMJ.

1 Introduction

Chronic pain is a common disabling ailment among men and women of all races and ethnicities (Stewart, Ricci, Chee, Morganstein, & Lipton, 2003) affecting 41% and 37% of the population in underdeveloped and developed countries, respectively (Tsang et al., 2008). In the US population alone, chronic pain affects 13% of the working population and costs an estimated $61 billion in lost productivity (Hardt, Jacobsen, Goldberg, Nickel, & Buchwald, 2009; Stewart, Ricci, Chee, Morganstein, & Lipton, 2003). There are male-specific chronic pain conditions, such as chronic prostatitis (Zhang et al., 2015), while some chronic pain conditions like gout (Higgins et al., 2017), ankylosing spondylitis (Exarchou et al., 2015), and cluster headaches (Exarchou et al., 2015) are more prevalent in men. Female-specific chronic pain conditions include endometriosis (Mehedintu, Plotogea, Ionescu, & Antonovici, 2014), vulvodynia (Hoffstetter & Shah, 2015), and

menstrual pain, while those that are more prevalent in women are fibromyalgia, irritable bowel syndrome, temporomandibular joint disorders (TMD), Raynaud's syndrome, rheumatoid arthritis, multiple sclerosis, and migraine (Wei, Yuan Ong, & Goadsby, 2018).

Further, there is a gender disparity not only in the predominance of pain disorders reported in women but also pain perception. Women report a greater sensitivity to pain (Fillingim, King, Ribeiro-Dasilva, Rahim-Williams, & Riley, 2009; Kim et al., 2013; Rosseland & Stubhaug, 2004), lower pain threshold, and less tolerance to pain (Fillingim, King, Ribeiro-Dasilva, Rahim-Williams, & Riley, 2009; IOM, 2011). This gender disparity in increased pain and/or pain sensitivity may be attributed to ovarian hormones. In support, pain sensitivity or intensity also varies during the menstrual cycle (Craft, 2007; Marcus, 1995; Martin, 2009; Riley, Robinson, Wise, & Price, 1999; Sherman & LeResche, 2006; Teepker, Peters, Vedder, Schepelmann, & Lautenbacher, 2010; Unruh, 1996). Exogenous ovarian hormones also affect pain such that oral contraceptives, which result in more constant hormone levels, can improve pain symptoms (Coffee, Sulak, & Kuehl, 2007; Craft, 2007; Sulak, Willis, Kuehl, Coffee, & Clark, 2007). However, exogenous hormones also increase pain sensitivity. Transgender individuals who undergo physical transition from male to female are usually prescribed a dose of estrogen equivalent to the recommended dose for hormone replacement therapy (HRT) in postmenopausal women (Moore, Wisniewski, & Dobs, 2003). These individuals

report an increase in pain, whereas, the individuals that transition physically from female to male report a significant improvement in pre-existing pain conditions (Aloisi et al., 2007).

Estrogen can be pronociceptive (Bi et al., 2017; Kou et al., 2011; Pratap et al., 2015; Ralya & McCarson, 2014; Wu et al., 2010; Zhang, Xiao, Zhang, Zhao, & Zhang, 2012; Zhang, Lu, Zhao, & Zhang, 2012) and can upregulate inflammatory mediators (Kou et al., 2011; Puri, Bellinger, & Kramer, 2011). Antinociceptive properties of estrogen have also been reported (Favaro-Moreira, Torres-Chavez, Fischer, & Tambeli, 2009; Fischer et al., 2008; Kramer & Bellinger, 2013). Progesterone and its metabolite, allopregnanolone, on the other hand, have well-documented anti-inflammatory (Coronel, Labombarda, De Nicola, & Gonzalez, 2014; Coronel et al., 2016; Garay et al., 2012; Grandi et al., 2016; He, Evans, Hoffman, Oyesiku, & Stein, 2004; Labombarda et al., 2011; VanLandingham, Cekic, Cutler, Hoffman, & Stein, 2007) and antinociceptive effects on neuropathic pain (Afrazi & Esmaeili-Mahani, 2014; Charlet, Lasbennes, Darbon, & Poisbeau, 2008; Coronel, Labombarda, Villar, De Nicola, & Gonzalez, 2011; Huang et al., 2016; Jarahi, Sheibani, Safakhah, Torkmandi, & Rashidy-Pour, 2014; Kawano et al., 2011; Liu et al., 2014). Progesterone and allopregnanolone have been reported to reduce neuropathic pain in animal models of chemotherapy-induced neuropathy (Meyer, Patte-Mensah, Taleb, & Mensah-Nyagan, 2010, 2011), sciatic nerve crush or constriction (Coronel, Labombarda, Villar, De Nicola, & Gonzalez, 2011; Huang et al., 2016; Roglio et al.,

2008), diabetic-neuropathy (Afrazi & Esmaeili-Mahani, 2014; Leonelli et al., 2007), and trigeminal nerve root demyelination (Kim et al., 2012).

One major pain disorder that is more prevalent in women is TMD pain. TMD affects approximately 10% of the population (LeResche, 1997) and is more prevalent in women (Bartley & Fillingim, 2013; Berkley, 1997; Isong, Gansky, & Plesh, 2008; Manson, 2010; Unruh, 1996), which account for 75% of all cases reported (Macfarlane, Glenny, & Worthington, 2001). TMD is a musculoskeletal orofacial pain disorder than may result from traumatic facial injuries, bruxism, or disk derangement (Liu & Steinkeler, 2013). The most common side effect of TMD is myofascial pain and inflammation; however, limitations in jaw movement, which affects speech and eating, is also common (List & Jensen, 2017; Liu & Steinkeler, 2013). While the causes of TMD are well established, the underlying mechanisms that make TMD pain more prevalent and severe in women is unclear, but the ovarian hormones estrogen and progesterone have been implicated. TMD pain intensifies at the onset of puberty, is highest during child-bearing years, and dissipates pregnancy and after menopause (LeResche, Mancl, Sherman, Gandara, & Dworkin, 2003). TMD pain has been reported to reemerge in some post-menopausal women undergoing HRT, particularly, estrogen replacement therapy (LeResche, Saunders, Von Korff, Barlow, & Dworkin, 1997; Wise, Riley, & Robinson, 2000).

Estrogen appears to be a key modulatory ovarian hormone that contributes to TMD pain. In animal models, estrogen enhances temporomandibular joint (TMJ) nociception (Bereiter, Okamoto, & Bereiter, 2005; Bi et al., 2017; Cairns, Sim, Bereiter, Sessle, & Hu, 2002; Flake, Bonebreak, & Gold, 2005; Kou et al., 2011; Kou et al., 2014; Okamoto, Bereiter, Thompson, Tashiro, & Bereiter, 2008), although the opposite has also been reported (Fischer et al., 2008). In support of a pronociceptive role, estrogen upregulates pro-inflammatory cytokines in the TMJ (Kou et al., 2011; Kou et al., 2014; Kou et al., 2015; Kramer & Bellinger, 2013; Puri, Bellinger, & Kramer, 2011; Xue et al., 2018; Yun, Chae, & Lee, 2008). Estrogen can also upregulate $GABA_A$ receptor subunit expression in the trigeminal ganglia (Puri, Bellinger, & Kramer, 2011) and voltage-gated sodium channels in the trigeminal ganglia resulting in hyperalgesia (Bi et al., 2017). Estrogen treatment decreases action potential thresholds in TMJ afferents (Flake, Bonebreak, & Gold, 2005) and increases reflex jaw muscle activity that was reduced by ovariectomy (Cairns, Sim, Bereiter, Sessle, & Hu, 2002). At second order neurons, estrogen increases neural activity and excitability within the trigeminal nucleus caudalis of the medullary spinal cord (Bereiter, Okamoto, & Bereiter, 2005; Okamoto, Bereiter, Thompson, Tashiro, & Bereiter, 2008).

While estrogen seems to have a complicated but predominantly pronociceptive role, the contribution of progesterone has been largely overlooked in research on TMD. It has been reported that both estrogen and progesterone

decrease formalin and glutamate-evoked TMJ nociceptive behaviors and progesterone reduces pro-inflammatory cytokines in the TMJ (Xue et al., 2017). Of the studies reporting the effects of estrogen and progesterone on TMD, no studies have examined the effects of ovariectomy on established mechanical allodynia at the inflamed TMJ and then re-examined sensory thresholds following the re-introduction of estrogen and progesterone. Therefore, we hypothesized that removal of endogenous ovarian hormones would attenuate inflammatory allodynia at the TMJ, which would return following estrogen treatment. We further hypothesized that progesterone and its metabolite allopregnanolone would attenuate the return of inflammatory orofacial mechanical allodynia in female rats.

2 Materials and Methods

2.1 Subjects

A total of 77 adult Sprague Dawley female rats (150-175 grams; Charles River Laboratories, Wilmington, MA) were used in these experiments. Rats were housed 2 per cage in a colony room with a 12:12 hour light:dark cycle (lights on at 8 a.m.). Food and water were available ad libitum. Rats were allowed one week to acclimate to the facility before experiments began. Vaginal lavages were conducted between 9 a.m. and 11 a.m. for 10 days or 2 consecutive cycles to ensure rats were cycling properly. Estrus was determined by the predominance of cornified epithelial tissue and proestrus was determined by the predominance of nucleated epithelial tissue.

Diestrus I was differentiated from diestrus II by the presence of leukocytes. All studies were approved by Texas Woman's University Institutional Animal Care and Use Committee. Experiments conformed to federal guidelines and the committee for Research and Ethical Issues of the International Association for the Study of Pain.

2.2 Drugs

Stock solutions of estradiol benzoate (E2), progesterone (P4), and 5α-Pregnan-3α-ol-20-one (allopregnanolone; Sigma Aldrich, St. Louis, MO) were dissolved in sesame seed oil (Sigma Aldrich, St. Louis, MO), diluted prior to injection, and administered subcutaneously (s.c).

2.3 Ovariectomy

Female rats were deeply anesthetized (induction 3%; maintenance 2.5%) with inhalation of gas (isoflurane, USP, Henry Schein Animal Health, Dublin, OH) anesthesia. Topical lidocaine was applied before a single incision was made to the anterolateral abdominal area. The abdominal muscle was cut, ovaries were ligated with 3-0 Vicryl sutures, and excised. Abdominal muscle was sutured with 3-0 Vicryl sutures and the epidermal layer was stapled with an Autoclip Wound Closing System (Braintree Scientific, Braintree, MA, USA). Immediately before and 24 hours following surgery, animals were administered the antibiotic Baytril (0.02 ml of a 22.7 mg/kg solution leading to approximately 4.4 mg/kg dose) intramuscularly (i.m.). The analgesic Rimadyl (0.03 ml of a 50 mg/kg solution leading to

approximately 2.5 mg/kg dose) was administered subcutaneously (s.c.) immediately following surgery. Rats that received sham surgery received the same procedural manipulations and treatments except for removal of ovaries. Rats were allowed two weeks recovery from surgery and for the elimination of endogenous ovarian hormones.

2.4 Temporomandibular Joint Inflammation

Complete Freund's Adjuvant (30 µl; CFA; mycobacterium tuberculosis; Sigma Aldrich) was dissolved 1:1 in saline solution and injected under brief isoflurane gas anesthesia into the intra-articular area of the TMJ. The TMJ area was palpated for the TMJ, confirmed by movement of the mandible, then the needle was directed to the joint and injected with CFA using a 30-gauge needle.

2.5 Behavior Testing

Behavior was tested before CFA injections, 24 hr after CFA injections, then 2 weeks after OVX. Von Frey filaments (North Coast Medical Inc, Gilroy, CA) were utilized to test the force to withdrawal threshold as a measure of mechanical allodynia at the cutaneous tissues surrounding the inflamed TMJ, as previously reported (Ren, 1999). For this test, a starting filament was first applied to the TMJ; 2.0-gram for non-inflamed tissues and 0.16-gram filament for inflamed tissue (Ren, 1999; Villa et al., 2010). If no response was observed, 30 seconds later the next thickest filament was applied, and so on until a withdrawal response was observed. If a withdrawal

response was observed with the starting filament, thirty seconds later the next thinnest filament was applied, and so on until no withdrawal response was observed. The filament size that produced at least three responses was recorded as the threshold grams of pressure required to elicit a withdrawal response as a measure of mechanical allodynia.

2.6 Hormone Treatments

To test the role of progesterone (P4), animals received one of the following hormone treatments s.c. every day for 5 days: (a) daily estradiol benzoate (E2) 50 μg/kg, (b) daily P4 (16 mg/kg, 16 μg/kg, or 16 ng/kg), (c) daily E2 and P4 (16 mg/kg, 16 μg/kg, or 16 ng/kg), (d) daily E2 and intermittent P4 (days 1, 3, and 5), or (e) vehicle (sesame seed oil) control. To test the effects of allopregnanolone, a separate group of rats received one of the following hormone treatments: (a) daily E2 (50 μg/kg), (b) daily allopregnanolone (0.16 mg/kg), (c) daily E2 and continuous allopregnanolone, (d) daily E2 and intermittent allopregnanolone (days 1, 3, and 5), or (e) vehicle control. Mechanical allodynia was reassessed one hour after injection for both experiments. The dose of allopregnanolone was chosen from a previous study reporting attenuation of mechanical allodynia in a rat model of post-operative neuropathic pain (Fujita, Fukuda, Sato, Takasusuki, & Tanaka, 2018).

2.7 Data Analysis

Behavioral data were presented as the mean ± standard error of the mean of the force in grams (g) required to elicit a withdrawal as a measure of the degree of mechanical allodynia. Data were analyzed by one-way analysis of variance (ANOVA) and two-way repeated measures ANOVA using GraphPad Prism 7 with time as the repeated measure and treatment as the independent factor. Tukey's post hoc analysis was conducted. Statistical significance was tested at $p \leq 0.01$. Hormone treatment data over 5 days is presented per day for clarity.

3 Results

3.1 Ovariectomy reverses CFA-evoked mechanical allodynia in the TMJ

Baseline mechanical threshold was measured before and after CFA injections then again two weeks after ovariectomy (OVX) or sham surgery (see timeline Figure 1A). There was a significant interaction between the treatment groups across time [$F(2,44) = 20.6$; $p \leq 0.01$]. CFA evoked a significant reduction in the force to withdrawal in all rats ($p \leq 0.01$; Figure 1B). Following OVX, the force required to elicit withdraw (in grams) was significantly greater in ovariectomized rats (closed bars; $p \leq 0.01$) compared to sham rats which remained allodynic (open bars). TMJ inflammation did not cause weight loss as animal weight 2 weeks post-surgery was significantly greater than animal weight prior to CFA injections [$F(1.859,117.1) = 441.4$; $p \leq 0.01$] (data not shown).

3.2 E2 (50 μg/kg) treatment elicits return of CFA-evoked mechanical allodynia in the TMJ

Following OVX, rats received five days of hormone treatment (see treatment groups Figure 1A) and mechanical allodynia was reassessed one hour following the last injection on day 5. There was a significant effect of treatment [$F(3,18) = 22.92$; $p \leq 0.01$]. Ovariectomized rats that received daily estradiol benzoate (E2) with daily progesterone (P4) (mid gray bars) retained significantly higher mechanical thresholds ($p \leq 0.01$), while rats that received only daily E2 (light gray bars) displayed similar mechanical allodynia to sham animals that received vehicle injections (open bars; $p > 0.01$; see Figure 1C). Rats that received daily E2 and P4 every other day (dark gray bars) also displayed similar mechanical allodynia to sham animals that received vehicle injections (open bars; $p > 0.01$; see Figure 1C).

3.3 Progesterone treatment rapidly protects against E2-elicited return of CFA-evoked mechanical allodynia in the TMJ

We then repeated the experiment with an altered timeline to assess whether the observed effects of P4 on mechanical allodynia occurred prior to day 5 of the treatments. Similar to Figure 1B, we found a significant effect of treatment on mechanical allodynia [$F(1, 76) = 42.8$; $p \leq 0.01$]. CFA again evoked a significant reduction in the force to withdrawal in all rats ($p \leq 0.01$; see Figure 2B). In rats that were then ovariectomized, the force to withdraw returned to baseline levels (closed

bars; $p \leq 0.01$) and was significantly greater than the sham surgery rats that remained allodynic (open bars; $p > 0.01$). There was a significant effect of treatment [$F(5,32) = 282.9$; $p \leq 0.01$], but not time [$F(1.9,61.5) = 1.931$; $p > 0.01$]. On day 1, there was a significant effect of treatment one hour following the first hormone treatment [$F(5,32) = 83.40$; $p \leq 0.01$; see Figure 2C]. We found significantly lower mechanical thresholds in ovariectomized females that received daily E2 (light gray bars; $p < 0.01$) and the sham rats that received vehicle treatment (open bars; $p \leq 0.01$) when compared to all other groups. There was also a significant effect of treatment on day 3 one hour following hormone injections [$F(5,32) = 98.61$; $p \leq 0.01$; see Figure 2D] with significantly lower mechanical thresholds in ovariectomized females that received daily E2 (light gray bars; $p \leq 0.01$) and the sham rats that received vehicle treatment (open bars; $p \leq 0.01$) when compared to all other groups. We did not observe a decrease in mechanical threshold in ovariectomized rats treated with P4 ($p > 0.01$), ovariectomized rats treated with E2 and P4 ($p > 0.01$), or ovariectomized rats treated with E2 and P4 every other day ($p > 0.01$) compared to sham control (open bars) and E2-treated rats (light gray bars). There was also a significant effect of treatment observed one hour after the last injection on day 5 [$F(5, 32) = 161.6$; $p \leq 0.01$; see Figure 2E]. Sham females that received vehicle injections (open bars) and ovariectomized females that received daily E2 (light gray bars) displayed a significantly lower force to withdraw compared to ovariectomized rats that received vehicle (crossed bars), daily E2 and

P4, daily P4 alone, or daily E2 with P4 injected every other day ($p \leq 0.01$). There was no significant difference between sham controls (open bars) and ovariectomized females treated with E2 (crossed bars; $p > 0.01$). No mechanical allodynia was observed in any group receiving P4 compared to sham or E2 injected rats ($p > 0.01$). On all three days, all rats receiving P4 treatment were similar to rats receiving vehicle treatment (crossed bars; $p > 0.01$).

3.4 A lower progesterone dose (16 µg/kg), but not (16 ng/kg), protects against E2-elicited return of CFA-evoked mechanical allodynia in the TMJ

We next tested whether a lower dose of P4, 16 µg/kg, could also protect against the E2-elicited return of mechanical allodynia. Using the same treatment paradigm as Figure 2A, we measured mechanical allodynia one hour after hormone injections on day 1, day 3, and day 5. There was a significant effect of treatment [$F(5,35) = 39.1$; $p \leq 0.01$], but not time [$F(1.9,67.9) = 0.77$; $p > 0.01$]. As the treatment effect was again the same on each day, we compared the treatment groups within each day. There was a significant effect of treatment with 16 µg/kg P4 on day 1 [$F(5,35) = 18.65$; $p \leq 0.01$; see Figure 3A], day 3 [$F(5,35) = 11.76$; $p \leq 0.01$; see Figure 3B], and day 5 [$F(5,35) = 26.13$; $p \leq 0.01$; see Figure 3C] of the treatment regimen. On each day, we observed a significantly lower mechanical threshold in the rats receiving daily E2 (light gray bars; $p \leq 0.01$) and sham controls (open bars; $p \leq 0.01$) compared to ovariectomized rats receiving vehicle (crossed bars) and all P4

treated rats with or without E2. There was no significant difference in CFA-evoked mechanical allodynia between sham controls (open bars) and ovariectomized rats receiving only E2 (light gray bars) on any treatment day tested ($p > 0.01$) and no mechanical allodynia was observed between any groups receiving P4 ($p > 0.01$).

We then tested whether an even lower dose of P4, 16 ng/kg, could also protect against the E2-elicited return of mechanical allodynia. There was a significant effect of treatment [$F(5,32) = 315.0$; $p \leq 0.01$], but not time [$F(1.9,62.2) = 1.3$; $p > 0.01$]. As the treatment effect was again the same on each day, we compared the treatment groups within each day. There was a significant effect of treatment on day 1 [$F(4, 27) = 162.2$; $p \leq 0.01$; see Figure 3D], day 3 [$F(4, 27) = 120.7$; $p \leq 0.01$see Figure 3E], and day 5 [$F(4, 27) = 268.2$; $p \leq 0.01$; see Figure 3F]. On each testing day, we observed a significantly lower force to withdraw in sham rats (open bars) compared to ovariectomized rats with vehicle treatments (crossed bars; $p \leq 0.01$) and rats receiving daily P4 only ($p \leq 0.01$). Significantly lower mechanical thresholds were also observed in ovariectomized rats treated with daily E2 (light gray bars) or in combination with daily or interrupted 16 ng/kg P4 compared to ovariectomized rats with vehicle treatments (crossed bars; $p \leq 0.01$) and compared to ovariectomized rats with only P4 treatment ($p \leq 0.01$).

3.5 Allopregnanolone (0.16 mg/kg) rapidly protects against E2-elicited return of CFA-evoked mechanical allodynia in the TMJ on treatment day 1, but not day 3 or day 5

The same behavior testing paradigm was used here, but allopregnanolone (AP) was injected instead of P4 and mechanical allodynia was detected on day 1, day 3, and day 5 (Figure 4A). We found a significant interaction between treatment groups across time [$F(10,62) = 39.1$; $p \leq 0.01$]. There was a significant effect of treatment [$F(5,31) = 295.6$; $p \leq 0.01$] and a significant effect of time [$F(2,62) = 66.2$; $p \leq 0.01$]. When comparing treatment groups within each day, there was a significant effect of treatment on day 1 [$F(5,31) = 47.54$; $p \leq 0.01$; see Figure 4B] one hour after hormone injections. The force to withdraw was significantly lower in ovariectomized rats treated daily with E2 (light gray bars; $p \leq 0.01$) and the sham controls (open bars; $p \leq 0.01$) when compared to ovariectomized females receiving vehicle (crossed bars), daily AP, daily E2 and AP, or daily E2 and AP every other day. No mechanical allodynia was observed in any group receiving AP compared to sham (open bars) or E2 injected rats (light gray bars; $p > 0.01$). There was also a significant effect of treatment on day 3 [$F(5,31) = 1762$; $p \leq 0.01$] and day 5 [$F(5,31) = 191.7$; $p \leq 0.01$]. However, on day 3 (Figure 4C) and day 5 (Figure 4D), mechanical threshold was significantly lower in all ovariectomized females receiving daily E2 with or without AP compared to receiving only AP ($p \leq 0.01$). Similar to day 1, rats

receiving daily AP did not display allodynia on day 3 or day 5 and were similar to vehicle treated rats ($p > 0.01$).

4 Discussion

Temporomandibular joint disorder (TMD) pain is a hallmark pain disorder more prevalent in women that is greatest during the reproductive years, dissipates after menopause (LeResche, Mancl, Sherman, Gandara, & Dworkin, 2003), and can reemerge with estrogen replacement therapy (LeResche, 1997; Wise, Riley, & Robinson, 2000). Progesterone and its metabolite allopregnanolone have anti-inflammatory and antinociceptive properties [for review see (Coronel, Labombarda, & Gonzalez, 2016)], while estrogen appears to be either pronociceptive (Kou et al., 2011; Pratap et al., 2015; Ralya & McCarson, 2014; Wu et al., 2010) or antinociceptive (Fischer et al., 2008; Kramer & Bellinger, 2013). Here we hypothesized that ovariectomy would attenuate inflammatory allodynia in the rat temporomandibular joint (TMJ) and that both progesterone and allopregnanolone would attenuate the reemergence of inflammatory TMJ allodynia in female rats. Overall, we report that (1) ovariectomy attenuated CFA-evoked mechanical allodynia at the TMJ, (2) estrogen treatment triggered the reemergence of mechanical allodynia which was attenuated by co-treatment with progesterone at 16 mg/kg and 16 μg/kg, but not 16 ng/kg, and (3) the progesterone metabolite was

also able to attenuate E2-evoked reemergence of allodynia, but only at the first injection day.

CFA injection at the TMJ triggered significant mechanical allodynia as measured by von Frey filaments, similar to previous studies (Guo et al., 2010; Ren, 1999; Villa et al., 2010). A limitation of this method is that deep pain in the joint is not detected; however, our data and previous studies indicate that mechanical allodynia can be readily detected at the cutaneous tissues surrounding the joint. Thus, while CFA-injection at the TMJ provides an inflammatory pain model, it may not fully capture the TMJ damage and resulting deep pain in the joint observed in the clinic. The repeated open-mouth procedure to induce TMJ dysfunction and orofacial mechanical allodynia (Wang et al., 2018) may provide a more clinically-relevant model to utilize to examine the potential mechanisms underlying the role of estrogen and progesterone on TMD pain. This model, however, does not produce persistent pain required for our experimental design. Future studies could integrate CFA- or carrageenan-evoked joint inflammation into the open-mouth procedure to create more persistent TMD-like pain conditions.

Following ovariectomy, mechanical sensitivity subsided to basal levels while sham animals remained allodynic, in concurrence with previous findings (Wu et al., 2010). While mechanical sensitivity decreased, the rats from that study did not return to basal levels of sensitivity, which is likely due to a difference in unilateral

versus bilateral CFA injections. Interestingly, when our rats were treated with estrogen a reemergence of mechanical allodynia was observed, supporting a pronociceptive role of estrogen in the TMJ, and similar to previous reports (Fejes-Szabo et al., 2018; Kou et al., 2011; Wang et al., 2013; Zhang, Lu, Zhao, & Zhang, 2012). In opposition, estrogen treatment can also attenuate CFA-induced TMJ nociception (Kramer & Bellinger, 2009). The opposing reports on estrogen's pronociceptive effects are likely due to differences in estrogen dose, timing of injections, and model used between studies. A low, continuously administered physiological dose of E2 (750 ng / 6 µl per day by a pump and an injection of 2.5 µg E2 every 5 days) administered prior to and during TMJ inflammation appears to be antinociceptive on orofacial pain (Kramer & Bellinger, 2009). We report that a higher E2 dose (50 µg/kg) administered after TMJ inflammation is pronociceptive. Together these data are interesting because they point to a possibility that consistent E2 is protective against orofacial pain when administered prior to and during TMJ inflammation, but large fluctuations in E2 administered after TMJ inflammation may enhance orofacial pain. This is supported by clinical reports that migraine is worsened during the late luteal and follicular phases, and during menstruation (Gupta, McCarson, Welch, & Berman, 2011; MacGregor et al., 2010; Shuster, Faubion, Sood, & Casey, 2011). Further, our data supports the clinical reports of women experiencing TMD pain reemergence while undergoing estrogen-replacement therapy. On the other hand, the same dose of E2 that was used in our

study was also reported to be antinociceptive in the formalin-inflamed rat TMJ model (Fischer et al., 2008). Estrogen may modulate formalin-induced inflammation differently than CFA-induced inflammation, resulting in either a pronociceptive or antinociceptive effect.

When estrogen was administered daily in the presence of progesterone, the development of allodynia was not observed. Others have also found that progesterone reduces the development of persistent pain in animal models of inflammatory pain (Ren, Wei, Dubner, Murphy, & Hoffman, 2000), diabetic neuropathy (Leonelli et al., 2007), and nerve injury (Coronel, Labombarda, Villar, De Nicola, & Gonzalez, 2011; Kim et al., 2012; Roglio et al., 2008; Roglio et al., 2009). Our data considered in the context of these studies indicate that progesterone may counter estrogen-evoked nociception. In support, a previous study reported that estrogen-evoked hyperalgesia was diminished when estrogen treatment was combined with progesterone (Ji, Tang, & Traub, 2005). Our findings support this protective effect of progesterone on estrogen-evoked pain and further we report that this occurs in the trigeminal system in a rat model of inflammatory pain at the TMJ.

Interestingly, we also found that the effects of both estrogen and progesterone occurred quickly. Evidence of estrogen-evoked reemergence of mechanical allodynia at the TMJ was observed on the first day of hormone

treatments one hour following injection. When progesterone was administered on an interrupted schedule instead of daily with estrogen, attenuation of mechanical allodynia occurred within one hour of injections and as early as the first day on the 5-day treatment schedule. These data indicate that progesterone has quick-acting, but not long-lasting actions on pain relief in the TMJ.

The optimal dose of progesterone used in different animal models, such as traumatic brain or spinal cord injury and neuropathic pain, is 16 mg/kg (Coronel, Labombarda, De Nicola, & Gonzalez, 2014; Coronel, Villar, Brumovsky, & Gonzalez, 2017; Labombarda et al., 2011; Pettus, Wright, Stein, & Hoffman, 2005). This dose reduces edema, improves cognitive function, and prevents neuronal loss (Cutler, Pettus, Hoffman, & Stein, 2005; Jarahi, Sheibani, Safakhah, Torkmandi, & Rashidy-Pour, 2014; Kasturi & Stein, 2009; Labombarda et al., 2009; Pettus, Wright, Stein, & Hoffman, 2005). While we found a protective effect of 16 mg/kg on pain, we also tested two lower doses of progesterone (16 μg/kg or 16 ng/kg) and observed rapid attenuation with the 16 μg/kg dose of progesterone but not with 16 ng/kg of progesterone. Others have also reported that timing, dosage, and duration of progesterone treatment is vital for attenuating nociceptive behaviors (Jarahi, Sheibani, Safakhah, Torkmandi, & Rashidy-Pour, 2014; Liu et al., 2014; Verdi, Jafari-Sabet, Mokhtari, Mesdaghinia, & Banafshe, 2013).

The acute effects of these two major gonadal hormones may be due to opposing activities on both pain and inflammation. Estrogen enhances nociception by upregulating inflammatory mediators (Kou et al., 2011; Pratap et al., 2015), upregulating injury-induced inflammatory processes (Flake, Hermanstyne, & Gold, 2006), modulating ion channel expression (Bi et al., 2017; Hu et al., 2012; Wu et al., 2010; Wu, Hao, Kou, Gan, & Ma, 2015), and increasing sensory neuron excitability in rats (Flake, Bonebreak, & Gold, 2005). Progesterone, on the other hand, decreases nociception by attenuating inflammatory microglial activation (Garay et al., 2012; Labombarda et al., 2011), inhibiting injury-induced upregulation of proinflammatory mediators (Coronel, Labombarda, De Nicola, & Gonzalez, 2014; Coronel et al., 2016; Garay et al., 2012; Grandi et al., 2016), and inhibiting ion channel activity (Johannessen, Fontanilla, Mavlyutov, Ruoho, & Jackson, 2011; Kelley & Mermelstein, 2011). Interestingly, progesterone, through actions at the intracellular progesterone receptor, inhibits the ability of estrogen to modulate gene expression and thus may underlie the attenuation of estrogen's effects on inflammatory mediators (Kraus, Weis, & Katzenellenbogen, 1995). So, it is possible in the present study that progesterone is counteracting estrogen's pronociceptive effects at the TMJ via inhibiting inflammatory mediators and preventing the estrogen-evoked upregulation of ion channel expression that triggers hypersensitivity. It is unclear where the site of action of gonadal hormones are in this system. Possibilities include direct action at trigeminal sensory neurons or

activity at the trigeminal nucleus caudalis where primary afferents enter the central nervous system. Proestrus estrogen levels upregulate neurotransmitter receptors and pro-inflammatory cytokines within the trigeminal ganglia, as well as, the superficial laminae of the upper cervical cord region (Vc/C_{1-2}) (Puri, Bellinger, & Kramer, 2011) and increase neural activity and excitability in the trigeminal nucleus caudalis of the medullary spinal cord (Bereiter, Okamoto, & Bereiter, 2005; Okamoto, Hirata, Takeshita, & Bereiter, 2003; Okamoto, Bereiter, Thompson, Tashiro, & Bereiter, 2008). Future studies are warranted to examine the effects of the hormone treatments utilized in the current study on the neural activity in the trigeminal nucleus caudalis.

Given the rapid effects of progesterone in the present study, progesterone may be acting at membrane progesterone receptors (mPR) rather than intracellular progesterone receptors (iPR). Progesterone can bind to iPR or mPR, while both progesterone and allopregnanolone bind to G protein coupled mPRs (Pang, Dong, & Thomas, 2013). However, given the current time course of progesterone's protective effect, it is unlikely that the iPR is involved. To date, 5 mPR subtypes have been identified (mPRα, mPRβ, mPRγ, mPRδ, and mPRε). Two of these receptors are coupled to a Gs protein increasing cyclic adenosine monophosphate (cAMP), while the other three are coupled to a Gi protein that decrease cAMP (Pang, Dong, & Thomas, 2013). These receptors are expressed throughout regions of the brain, such as thalamus, spinal cord, and medulla (Pang, Dong, & Thomas, 2013), as well as the

trigeminal ganglia (Manteniotis et al., 2013). Their role in pain in not clear, but inhibiting cAMP or adenylyl cyclase has analgesic or antinociceptive properties (Cheng et al., 2019; Shao et al., 2016). In the current study, it is possible that progesterone inhibits estrogen's effects on pain via mPRs that inhibit adenylyl cyclase.

Alternatively, progesterone's acute effects on pain may involve the sigma 1 receptor (Sig-1R). The Sig-1R is a non-opioid chaperone receptor located in the plasma membrane of the endoplasmic reticulum (Hayashi & Su, 2003) expressed in regions associated with pain regulation, such as dorsal root ganglia, spinal cord, thalamus, and rostroventral medulla of female mice (Sanchez-Fernandez et al., 2014) and the spinal cord, thalamus, and sciatic of male rats (Alonso et al., 2000). Sig-1R is reported in the trigeminal ganglia of male mice (Yoon, Kang, Kim, Kim, & Roh, 2015), but expression in female trigeminal sensory neurons currently unknown. Activation of Sig-1R elicits nociceptive responses, which can be reversed with SR1 antagonists (Entrena et al., 2016; Gris, Merlos, Vela, Zamanillo, & Portillo-Salido, 2014; Kim et al., 2008a; Parenti et al., 2014; Pyun, Son, & Kwon, 2014; Roh & Yoon, 2014; Tejada et al., 2014) or in Sig-1R knockout mice (de la Puente et al., 2009; Entrena et al., 2009) . Progesterone is a potent Sig-1R antagonist (Johannessen, Fontanilla, Mavlyutov, Ruoho, & Jackson, 2011; Zamanillo, Romero, Merlos, & Vela, 2013) and can inhibit nociception (Maurice, Urani, Phan, & Romieu, 2001; Maurice & Su, 2009; Ueda et al., 2001). In the current study, it is possible that

progesterone could be acting at the Sig-1R to counteract estrogen's effects on inflammatory orofacial allodynia. Further studies are warranted to determine if either mPRs or Sig-1R, and in what anatomical area, are involved in progesterone's protection effects.

Additionally, this rapid attenuation by progesterone may involve its metabolite, allopregnanolone. Allopregnanolone synthesis from progesterone involves two enzymes, 5α-reductase and 3α-hydroxysteroid oxidoreductase. The former converts progesterone to 5α-dihydroprogesterone (5α-DHP), while the latter converts 5α-DHP to allopregnanolone (Schumacher et al., 2014). Allopregnanolone has been shown to have antinociceptive effects. Allopregnanolone attenuates diabetes-induced neuropathy (Afrazi & Esmaeili-Mahani, 2014), postoperative pain (Fujita, Fukuda, Sato, Takasusuki, & Tanaka, 2018), inflammatory pain (Charlet, Lasbennes, Darbon, & Poisbeau, 2008; Ocvirk, Pearson Murphy, Franklin, & Abbott, 2008), sciatic nerve ligation nociception (Meyer, Venard, Schaeffer, Patte-Mensah, & Mensah-Nyagan, 2008), and chemotherapy-induced nociception (Meyer, Patte-Mensah, Taleb, & Mensah-Nyagan, 2011). In the present study, we observed a rapid attenuation in the reemergence of mechanical allodynia within one hour of injection on day 1. This is in agreement with previous studies that investigated the effects of allopregnanolone in animal models of neuropathic pain (Afrazi & Esmaeili-Mahani, 2014; Charlet, Lasbennes, Darbon, & Poisbeau, 2008; Fujita, Fukuda, Sato, Takasusuki, & Tanaka, 2018; Kawano et al.,

2011; Meyer, Venard, Schaeffer, Patte-Mensah, & Mensah-Nyagan, 2008; Meyer, Patte-Mensah, Taleb, & Mensah-Nyagan, 2011; Ocvirk, Pearson Murphy, Franklin, & Abbott, 2008; Svensson, Persson, Fitzsimmons, & Yaksh, 2013).

Surprisingly, allopregnanolone treatment did not attenuate the return of mechanical allodynia on day 3 or 5 of hormone treatment. This is not in agreement with a study that reported allopregnanolone suppressed diabetes-induced thermal hyperalgesia up to 7 weeks (Afrazi & Esmaeili-Mahani, 2014). The variance could be due to different dosage effects. Their study utilized high doses of allopregnanolone (5 mg/kg or 20 mg/kg) compared to our dose of 0.16 mg/kg. Perhaps a high dose of allopregnanolone is necessary to provide continuous attenuation of the return of orofacial mechanical allodynia after several days of repeated hormone treatment. It was previously reported that the most potent dose of allopregnanolone in reducing nociception is 0.16 mg/kg, but effects over time were not investigated (Ocvirk, Pearson Murphy, Franklin, & Abbott, 2008). It could be that allopregnanolone underlies protective effects on pain early on in treatment, but that progesterone's continual attenuation of the return of orofacial mechanical allodynia on the other days may involve a mechanism such as the sigma1 receptor. However, future studies are warranted to determine the reason for the observed effects of allopregnanolone in the current study on the reemergence of mechanical allodynia following estrogen treatment.

Allopregnanolone may have acute antinociceptive effects through the $GABA_A$ receptor. This is supported by reports that allopregnanolone decreases pain in neuropathy models (Afrazi & Esmaeili-Mahani, 2014; Huang et al., 2016). This effect may then be attenuated on day 3 and day 5 due to the development of tolerance to allopregnanolone. Repeated daily administration of allopregnanolone was reported to induce tolerance to allopregnanolone's anticonvulsant (Czlonkowska et al., 2001) and hypothermic properties (Palmer, Moyer, Crabbe, & Phillips, 2002). Also, a 90-minute exposure to allopregnanolone triggers an increase in allopregnanolone in brain regions important in tolerance development (Zhu, Birzniece, Backstrom, & Wahlstrom, 2004) [for review see (Turkmen, Backstrom, Wahlstrom, Andreen, & Johansson, 2011)]. Interestingly, prolonged exposure to allopregnanolone alters $GABA_A$ receptor subunits, resulting in a decrease in sensitivity to allopregnanolone (Turkmen, Lofgren, Birzniece, Backstrom, & Johansson, 2006; Turkmen, Wahlstrom, Backstrom, & Johansson, 2008; Zhu, Birzniece, Backstrom, & Wahlstrom, 2004). Alternatively, on days 3 and 5 estrogen may be further increasing the excitability of TMJ neurons (Flake, Bonebreak, & Gold, 2005), increasing inflammatory mediators, and increasing $GABA_A$ receptor in the trigeminal ganglia (Puri, Bellinger, & Kramer, 2011); all of which could be opposing protective effects of allopregnanolone. Based on these lines of evidence, studies examining potential effects of progesterone and allopregnanolone on the $GABA_A$ receptor in the trigeminal ganglia are needed to

understand the mechanism underlying the protective role of progesterone in our treatment paradigm.

Overall, we show that removal of the endogenous source of ovarian hormones after orofacial inflammation relieves mechanical allodynia in the TMJ of female rats. We also showed rapid attenuation of the return of mechanical allodynia by two different doses of progesterone and acute, rapid attenuation by allopregnanolone. These data suggest the allopregnanolone may provide short-term relief, whereas, progesterone may provide continual relief in the reemergence of TMD pain in post-menopausal women undergoing estrogen replacement therapy.

5 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6 Author Contributions

RSH contributed to experimental design, conducting experiments, data analysis and interpretation, and preparation of the manuscript. WB contributed to experimental design, conducting experiments, data analysis and interpretation, and approval of the manuscript. ST contributed to conducting experiments, data analysis and interpretation, and approval of the manuscript. LU contributed to experimental

design, data analysis and interpretation, and editing the manuscript. DA contributed to experimental design, data analysis and interpretation, and preparation of the manuscript.

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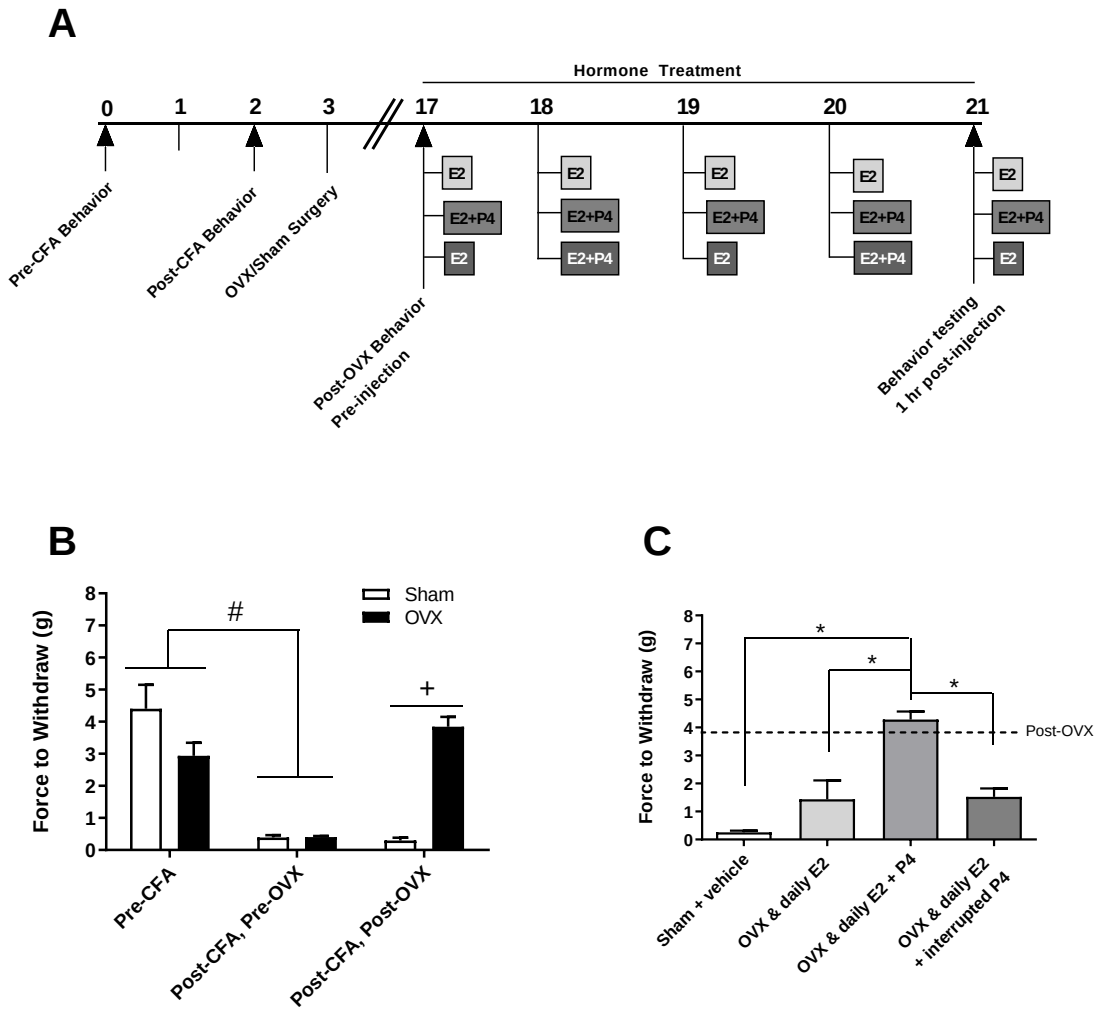

Figure 1. Effects of ovariectomy and gonadal hormone treatment on CFA-evoked mechanical allodynia in the inflamed rat temporomandibular joint.

(A) Experimental timeline of behavior testing, ovariectomy, and hormone treatments administered. Behavior testing was done 24-hrs prior to and following CFA injections followed by either ovariectomy (OVX; *n* = 19) or sham surgery (*n* = 5). Two-weeks later behavior testing occurred followed by hormone treatments.

Hormones were administered every day for 5 days except interrupted progesterone, which was administered on days 2 and 4. Behavior testing occurred one hour after hormone injection on day 5. (B) Bar graph showing CFA-evoked mechanical allodynia (pre-CFA vs post-CFA/pre-OVX) followed by sham (open bars) vs OVX (closed bars) surgery (post-CFA/post-OVX). (C) Effects of hormone treatment one hour after last treatment on Day 5 of a five-day hormone treatment regimen of daily estradiol (E2; $n = 5$), daily E2 and daily progesterone (E2+P4; $n = 7$), or daily E2 with interrupted P4 ($n = 5$) compared to sham treated with daily vehicle (sesame seed oil, open bars; $n = 5$). # indicates significant difference between pre-CFA and post-CFA/pre-OVX groups. + indicates significant difference between OVX and sham groups post-surgery. * indicates significant difference between hormone treatment groups. Statistical significance was tested at $p \leq 0.01$.

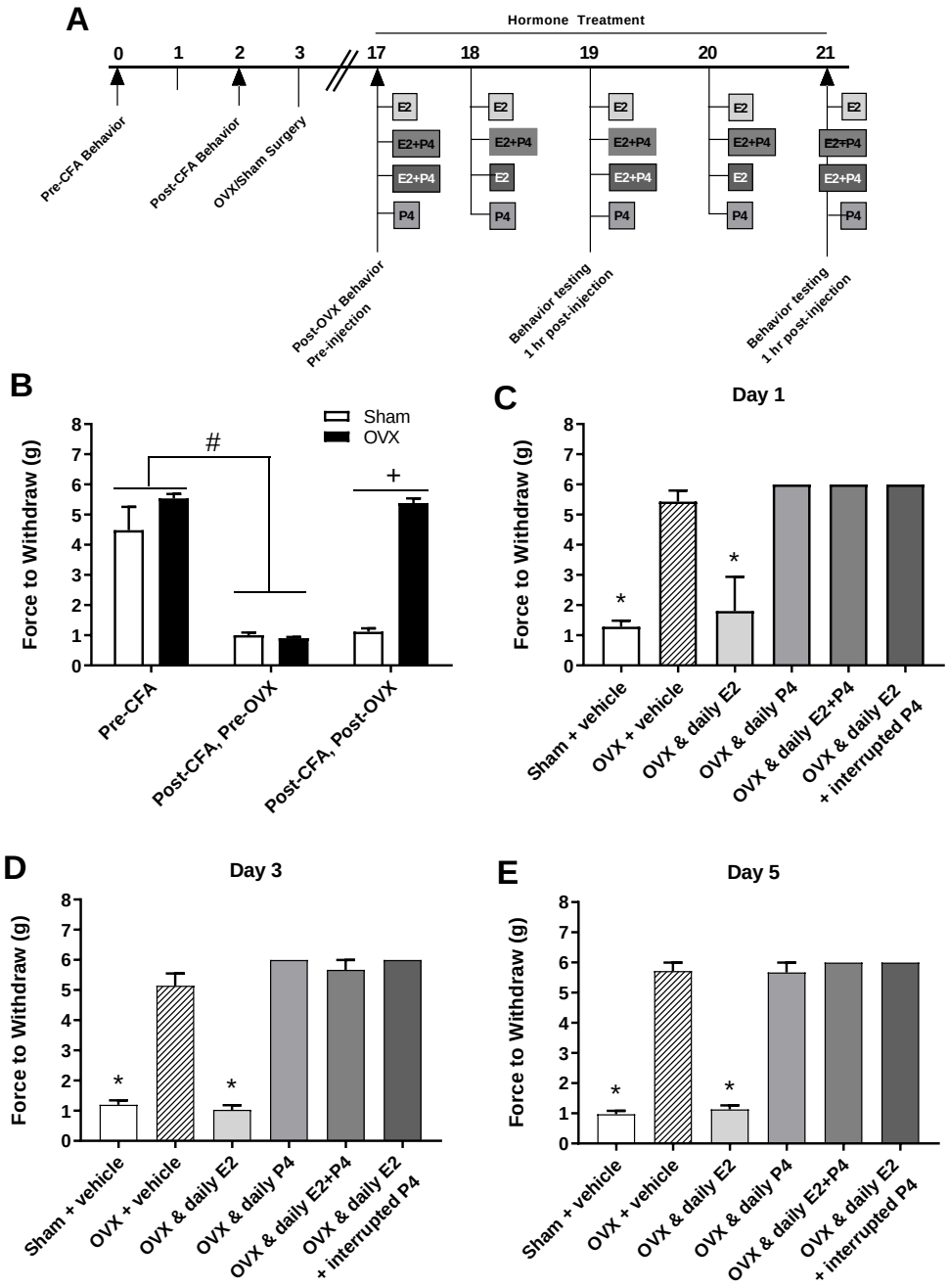

Figure 2. Effects of 16 mg/kg progesterone treatment on estrogen-exacerbated inflammatory mechanical allodynia in the inflamed rat temporomandibular joint.

(A) Experimental timeline of behavior testing, ovariectomy, and hormone treatments administered. Behavior testing and hormone treatments are different than experimental timeline seen in Figure 4.1. Behavior testing was done 24-hrs prior to and following CFA injections followed by either ovariectomy (OVX; closed bars) or sham surgery (open bars). Two-weeks later behavior testing occurred followed by hormone treatments. Hormones were administered every day for 5 days except interrupted progesterone, which was administered on days 1, 3, and 5. Behavior testing occurred one hour after hormone injection on day 1, day 3, and day 5. (B) Bar graph showing CFA-evoked mechanical allodynia (pre-CFA vs post-CFA/pre-OVX) followed by sham (open bars; $n = 7$) vs OVX (closed bars; $n = 7$) surgery (post-CFA/post-OVX). Mechanical threshold in OVX rats for day 1 (C), day 3 (D), and day 5 (E) one hour after last injection of daily vehicle (sesame seed oil; crossed bars; $n = 7$), daily estradiol (E2; $n = 6$), daily progesterone (P4; $n = 6$), daily E2 and daily P4 (E2+P4; $n = 6$), or daily E2 with interrupted P4 ($n = 6$) compared to sham treated with daily vehicle (open bars; $n = 7$). # indicates significant difference from pre-CFA and post-CFA/post-OVX groups. + indicates significant difference between OVX and sham groups post-surgery. * indicates significant difference compared to OVX & vehicle control group. Statistical significance was tested at $p \leq 0.01$.

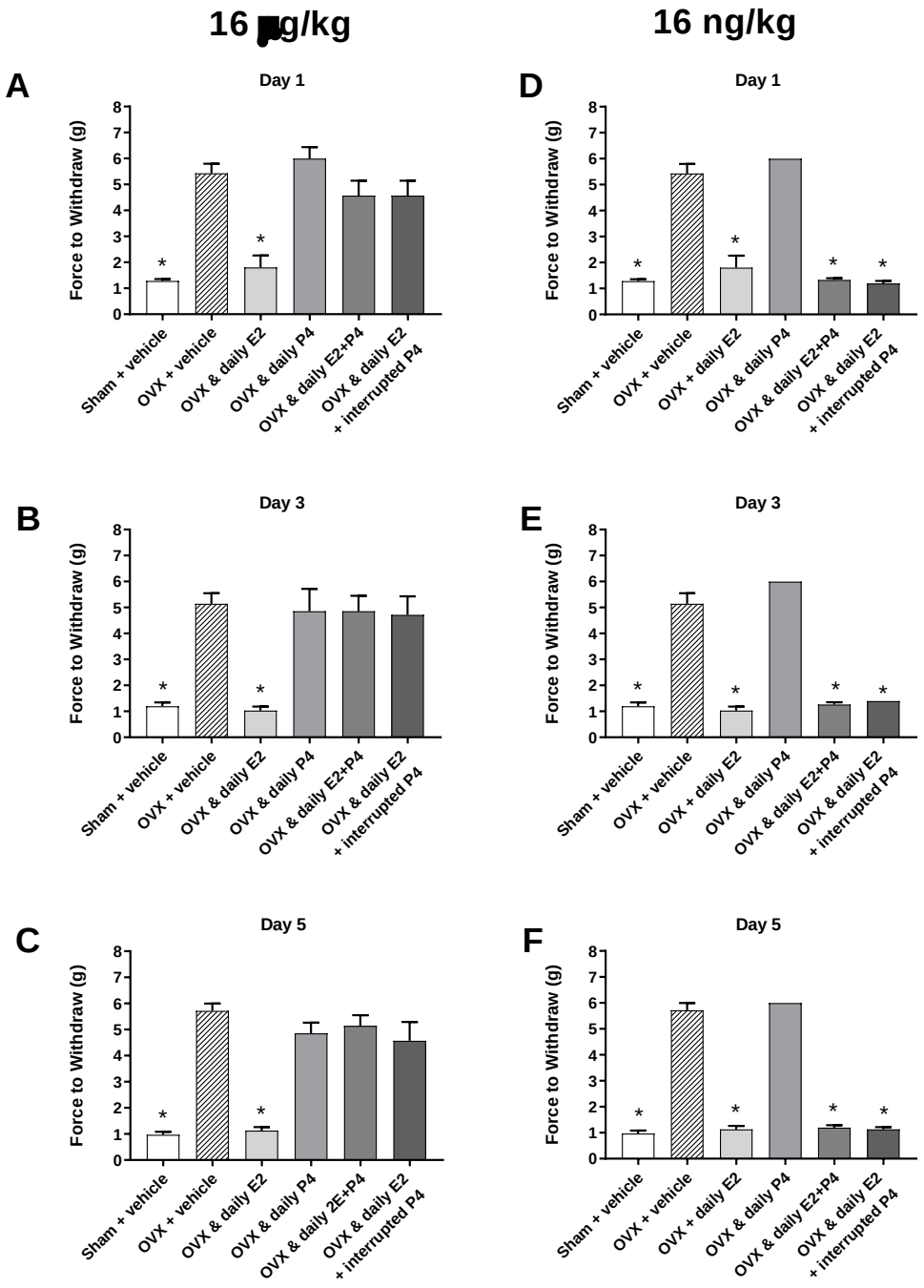

Figure 3. Effects of 16 µg/kg and 16ng/kg progesterone on estrogen-exacerbated inflammatory mechanical allodynia in the inflamed rat temporomandibular joint.

Experimental timeline of behavior testing, ovariectomy, and hormone treatments administered are the same as the timeline in Figure 2. Behavior testing was done 24-hrs prior to and following CFA injections followed by either ovariectomy (OVX) or sham surgery. Two-weeks later behavior testing occurred followed by hormone treatments. Hormones were administered every day for 5 days except interrupted progesterone, which was administered on days 1, 3, and 5. Behavior testing occurred one hour after hormone injection on day 5. Effects of 16 µg/kg of progesterone on mechanical threshold in OVX rats one hour after last injection of daily vehicle (sesame seed oil; crossed bars; n = 6), daily estradiol (E2; n = 6), daily progesterone (P4; n = 7), daily E2 and daily P4 (E2+P4; n = 7), or daily E2 with interrupted P4 (n = 7) compared to sham treated with daily vehicle (open bars; n = 7) on day 1 (**A**), day 3 (**B**) and day 5 (**C**). Effects of 16 ng/ kg of progesterone on mechanical threshold in OVX rats one hour after last injection of daily vehicle (sesame seed oil; crossed bars; n = 7), daily estradiol (E2; n = 6), daily progesterone (P4; n = 6), daily E2 and daily P4 (E2+P4; n = 6), or daily E2 with interrupted P4 (n = 6) compared to sham treated with daily vehicle (open bars; n = 7) on day 1 (**D**), day 3 (**E**), and day 5 (**F**) for 16 ng/kg of progesterone. * indicates significant difference from OVX & vehicle group. Statistical significance was tested at $p \leq 0.01$.

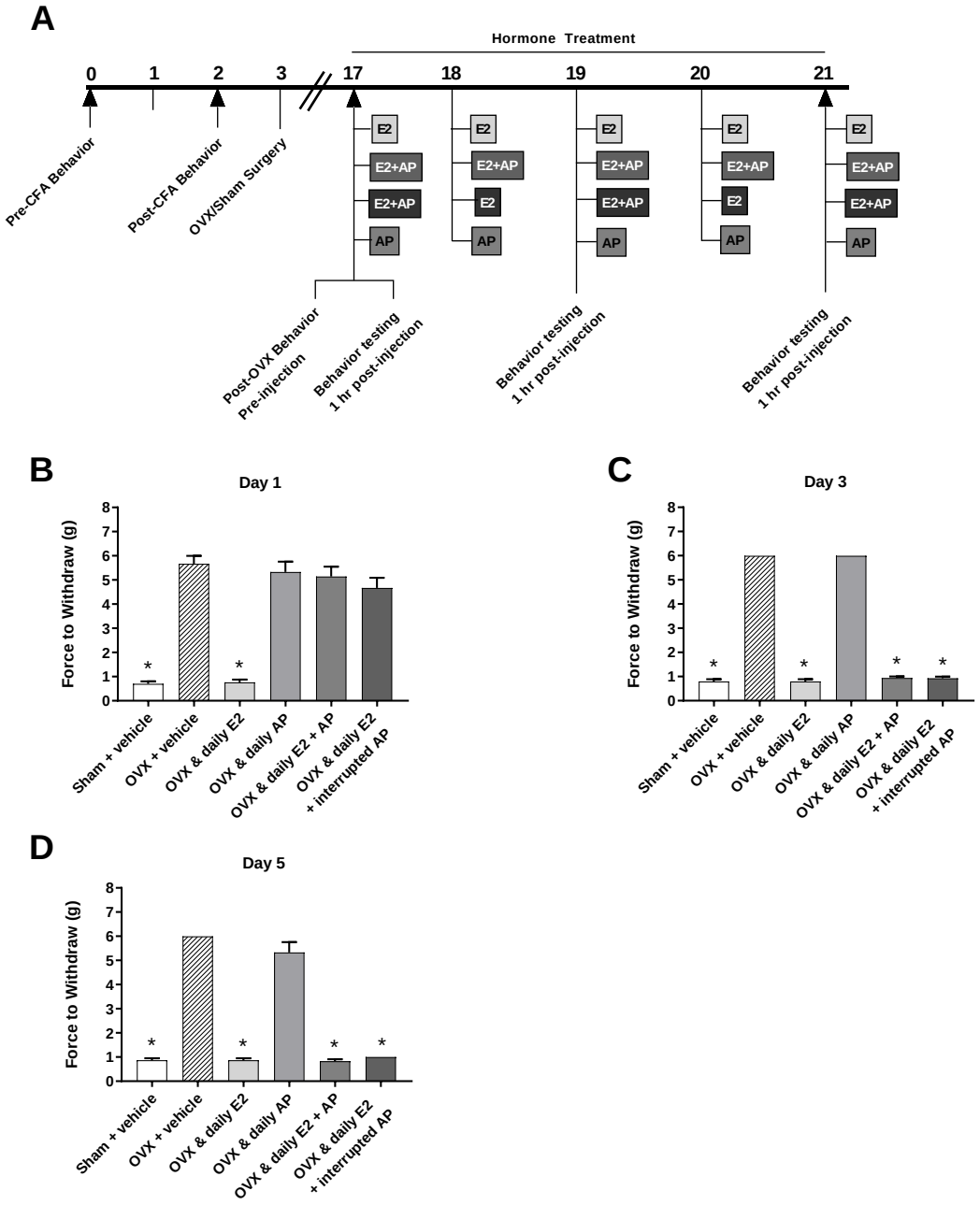

Figure 4. Effects of the progesterone metabolite allopregnanolone on estrogen-exacerbated inflammatory mechanical allodynia in the inflamed rat temporomandibular joint.

(A) Experimental timeline of behavior testing, ovariectomy, and hormone treatments administered. Behavior testing was done 24-hrs prior to and following CFA injections followed by either ovariectomy (OVX) or sham surgery. Two-weeks later behavior testing occurred followed by hormone treatments. Hormones were administered every day for 5 days except interrupted allopregnanolone, which was administered on days 1, 3, and 5. Behavior testing occurred one hour after hormone injection on day 5. Bar graphs showing mechanical threshold in OVX rats on day 1 (B), day 3 (C), and day 5 (D) one hour after last injection of daily vehicle (sesame seed oil; crossed bars; $n = 6$), daily estradiol (E2; $n = 6$), daily allopregnanolone (AP; $n = 6$), daily E2 and daily AP (E2+AP; $n = 7$), or daily E2 with interrupted AP ($n = 6$) compared to sham treated with daily vehicle (open bars; $n = 6$). * indicates significant difference from OVX & vehicle group. Statistical significance was tested at $p \leq 0.01$.

CHAPTER IV

SIGMA 1 RECEPTORS AND PROGESTERONE METABOLIZING ENZYMES, 5α-REDUCTASE AND 3α-HYDROXYSTEROID OXIDOREDUCTASE, ARE EXPRESSED IN THE TRIGEMINAL GANGLIA OF FEMALE RATS

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ABSTRACT

Orofacial pain disorders, such as migraine and temporomandibular joint disorder, are predominately experienced by women. Progesterone, a major ovarian hormone, has well known neuroprotective effects and attenuates pain sensitivity and increases pain thresholds. Previously, we have shown that progesterone attenuates the return of orofacial pain behaviors following hormone treatment. It

remains unknown what anatomical substrate underlies progesterone's activity in the trigeminal system. Progesterone has been reported to exert protective effects through actions at intracellular progesterone receptors (iPR), membrane-progesterone receptors (mPR), or sigma 1 receptors (Sig-1R). Of these, the iPR and Sig-1R have been reported to have a role in pain. Progesterone can also have antinociceptive effects through its metabolite, allopregnanolone. Two enzymes, 5α-reductase and 3α-hydroxysteroid oxidoreductase (3α-HSOR), are required for the metabolism of progesterone to allopregnanolone. We have previously reported that both progesterone and allopregnanolone can rapidly attenuate pain sensitivity, suggesting involvement of progesterone acting at Sig-1R, rather than iPR, and allopregnanolone acting at $GABA_A$ receptors. Progesterone's rapid effects attenuating orofacial pain may involve various mechanism; however, the anatomical substrate of progesterone's antinociceptive effects on orofacial pain is unclear. In the present study, we investigated whether Sig-1Rs are expressed in the trigeminal ganglia of cycling female rats and whether the two enzymes required for progesterone metabolism to allopregnanolone, 5α-reductase and 3α-hydroxysteroid oxidoreductase, are also present. Normal cyclicity in female Sprague Dawley rats was confirmed by vaginal lavages for 10 days and the phase of estrous cycle was recorded on the day of tissue collection. Female rats from each stage of the estrous cycle (n = 8 per stage) were rapidly decapitated and the trigeminal ganglia collected. Trigeminal ganglia were processed by either fluorescent immunochemistry (n = 4

per stage) or western blotting (n = 4 per stage) to both visualize the neuroanatomical localization and quantify the levels of Sig-1R, 5α-reductase, and 3α-hydroxysteroid oxidoreductase. Here we report that Sig-1Rs are expressed in nociceptive sensory neurons in the female rat trigeminal ganglia across the estrous cycle. Further, both enzymes involved in progesterone metabolism are present in the trigeminal ganglia of female rats across the estrous cycle. These data indicate that trigeminal sensory neurons could be an anatomical substrate for the reported antinociceptive activity of progesterone via Sig-1R and/or conversion to allopregnanolone.

Keywords: progesterone, trigeminal sensory neurons, allopregnanolone, sigma-1 receptor, 5α-reductase, 3α-hydroxysteroid oxidoreductase

1 Introduction

Orofacial pain is a general term used for pain conditions that affect structures of the head, face, neck, and oral cavity. These cranial and orofacial structures are innervated by trigeminal nociceptors that have cell bodies in the trigeminal ganglia and send nociceptive sensory input to the central nervous system. Pain signaled by the trigeminal nociceptors is common, often debilitating (Macfarlane, Blinkhorn, Davies, Kincey, & Worthington, 2003), and includes musculoskeletal, neurovascular, and neuropathic pain (Shaefer, Khawaja, & Bavia, 2018; Smith, Ceusters, Goldberg, & Ohrbach, 2011). In fact, orofacial pain affects 26% of the general population with the

majority of patients being women (Koopman et al., 2009; Macfarlane, Blinkhorn, Davies, & Worthington, 2003). Orofacial pain disorders include temporomandibular joint disorders (TMD) (Anastassaki & Magnusson, 2004; LeResche, 1997; Manfredini et al., 2011), cervicalgia including cervicogenic headaches (Haldeman & Dagenais, 2001), tension headaches (Buse et al., 2013; Lipton et al., 2007), trigeminal neuralgia (Siqueira, Teixeira, & Siqueira, 2009), burning mouth syndrome, which is predominantly middle-aged menopausal women (Zakrzewska, Forssell, & Glenny, 2005), and migraine (Lipton et al., 2007). Women also report greater pain sensitivity (Rosseland & Stubhaug, 2004), less tolerance, and a lower pain threshold than men (Fillingim, King, Ribeiro-Dasilva, Rahim-Williams, & Riley, 2009).

Importantly, women report variance in their orofacial pain across their menstrual cycle with pain sensitivity increasing in the late luteal phase and peaking during menses (Hellstrom & Anderberg, 2003; LeResche, Mancl, Sherman, Gandara, & Dworkin, 2003). Women that are pregnant or postmenopausal report a decrease in orofacial pain sensitivity, particularly TMD, with some postmenopausal reporting their TMD pain reemerges following HRT (LeResche, Saunders, Von Korff, Barlow, & Dworkin, 1997; LeResche et al., 2005; Wise, Riley, & Robinson, 2000). Preclinical studies also report a reduction in nociceptive responses in pregnant female rats following formalin injection into the TMJ (Arthuri, Gameiro, Tambeli, & de Arruda Veiga, 2005). Formalin or glutamate injection into the TMJ of female rats also results

in increased pain sensitivity during diestrus when 17β-estradiol levels begin to rise (Fischer et al., 2008). Despite the clear link between fluctuating gonadal hormones and TMD pain, the mechanisms underlying the effects of gonadal hormones on TMD pain remains elusive.

Estrogen and progesterone, female gonadal hormones, are primarily synthesized in the ovaries of females. These gonadal hormones are also neurosteroids as they can be locally synthesized in neurons and glial cells (Barakat, Oakley, Kim, Jin, & Ko, 2016; Schumacher et al., 2001), which allows these hormones to directly modulate both the central and peripheral nervous system. While estrogen has been reported to be both pronociceptive and antinociceptive in various pain models (Bi et al., 2017; Fischer et al., 2008; Flake, Bonebreak, & Gold, 2005; Kou et al., 2011; Kramer & Bellinger, 2013; Wu et al., 2010), the research literature exclusively reports that progesterone is anti-inflammatory and antinociceptive (Coronel, Labombarda, Villar, De Nicola, & Gonzalez, 2011; Ginanneschi et al., 2012; Kim et al., 2012; Leonelli et al., 2007; Meng, Barton, Goodney, Russell, & Mecum, 2019; Moazen, Taherianfard, Ahmadi Soleimani, & Norozpor, 2018; Roglio et al., 2008). We have recently reported that estrogen increases pain behaviors in a rat model of inflammatory TMD pain which is reversed with progesterone treatment (Hornung, Benton, Tongkhuya, Uphouse, & Averitt, under revision). Progesterone is also metabolized by 5α-reductase to 5α-dihydroprogesterone, which is then converted to the metabolite allopregnanolone by 3α-hydroxysteroid oxidoreductase

(3α-HSOR), thus progesterone's protective effects can occur through its metabolite allopregnanolone. Allopregnanolone is a positive allosteric modulator of the γ-aminobutyric acid type A ($GABA_A$) receptor, which underlies its antinociceptive properties (Gee, Bolger, Brinton, Coirini, & McEwen, 1988). Indeed, we recently reported that injection of allopregnanolone can attenuate pain behaviors in a rat model of inflammatory TMD pain (Hornung, Benton, Tongkhuya, Uphouse, & Averitt, under revision). In support, mechanical and thermal pain thresholds in spinal cord injury animals were reduced following either an intrathecal injection of Provera, a pharmacological inhibitor of 3α-HSOR activity, (Meyer, Venard, Schaeffer, Patte-Mensah, & Mensah-Nyagan, 2008) or siRNA knockdown 3α-HSOR (Patte-Mensah, Meyer, Schaeffer, & Mensah-Nyagan, 2010).

Despite the evidence that progesterone and allopregnanolone are antinociceptive and attenuate orofacial pain, it is currently unknown whether progesterone or allopregnanolone can act directly at trigeminal sensory neurons. Progesterone can exert protective effects through the iPR, mPR, Sig-1R, and its neuroactive metabolite allopregnanolone (Johannessen, Fontanilla, Mavlyutov, Ruoho, & Jackson, 2011; Pang, Dong, & Thomas, 2013; Singh & Su, 2013). Both iPRs and mPRs are widely expressed within the brain (Pang, Dong, & Thomas, 2013) and mPRs are expressed in the trigeminal ganglia (Manteniotis et al., 2013) but their role in nociception is not known. Progesterone also acts as an antagonist of the sigma 1 receptor (Sig-1R) (Johannessen, Fontanilla, Mavlyutov, Ruoho, & Jackson,

2011). Sig-1R is a non-opioid receptor located within the plasma membrane of the endoplasmic reticulum. Agonists for the Sig-1R elicit nociceptive responses (Entrena et al., 2016; Kim et al., 2008b; Pyun, Son, & Kwon, 2014; Ueda et al., 2001), which are reversed by antagonists (Cendan, Pujalte, Portillo-Salido, & Baeyens, 2005; Entrena et al., 2016; Garcia-Martinez et al., 2016; Kang et al., 2016; Parenti et al., 2014; Roh & Yoon, 2014; Son & Kwon, 2010; Ueda et al., 2001). Alternatively, progesterone may be metabolized locally in sensory neurons to allopregnanolone to inhibit pain via $GABA_A$ receptors expressed in trigeminal sensory neurons.

The current study was designed to determine whether Sig-1Rs and/or the enzymes involved in the conversion of progesterone to allopregnanolone are present in the nociceptive population of sensory neurons of the female rat trigeminal ganglia. As female rats may have variations in expression levels of membrane proteins when ovarian hormones fluctuate across the estrous cycle (Maguire, Stell, Rafizadeh, & Mody, 2005; Maguire & Mody, 2007), we also examined whether the expression of Sig-1Rs and the enzymes display plasticity in expression levels across diestrus, proestrus, and estrus. Here we utilized fluorescent immunohistochemistry, confocal microscopy, and western blotting techniques to uncover two available mechanisms underlying the effects of progesterone on orofacial pain.

2 Methods and Materials

2.1 Subjects

A total of 32 adult female Sprague Dawley rats (150-200 g; Charles River Laboratories) were used in these experiments. Rats were double-housed in a 12:12 hour light-dark cycle with lights on at 8 a.m. Food and water were available *ad libitum*. Rats were acclimated to the facility for one week before experiments began. All studies were approved by the Texas Woman's University Institutional Animal Care and Use Committee and conform to federal guidelines.

2.2 Vaginal Cytology

Vaginal lavages were conducted daily between 9 a.m. and 11 a.m. to confirm animals were cycling normally and to determine the phase of estrous cycle on the day of tissue collection. Proestrus was determined by the predominance of nucleated epithelial tissue and estrus was predominantly cornified epithelial tissue. Diestrus 1 was differentiated from diestrus 2 by the presence of leukocytes. Rats (n = 8 per stage) were rapidly decapitated between 9 a.m. and 11 a.m. and their trigeminal ganglia were removed. Tissues collected for western blots were stored at -80°C and tissue for immunofluorescent staining was stored in Tissue-Plus O.C.T (Optimal Cutting Temperature) Compound (SciGen Scientific, Gardenia, CA, USA) at -80 °C until further use.

2.3 Protein Extraction

Total protein extraction of bilateral trigeminal ganglia from rats that were in proestrus, estrus, diestrus 1, or diestrus 2 (n = 4 per stage) were homogenized in lysing matrix tubes (MP Biomedicals; Solon, OH, USA) with Pierce™ RIPA buffer (Thermo Scientific; Rockford, IL, USA) with Halt™ protease inhibitor cocktail (Thermo Scientific; Rockford, IL, USA) to prevent proteolysis. Tissue homogenization was performed for 10 seconds at 6.0 m/s for a total of three cycles, using VWR® homogenizer bead mill (Avantor; Radnor, PA, USA). Homogenates were then centrifuged at 13,000 rpm for 15 minutes at 4 °C. Supernatant was collected and protein concentration was determined by Pierce™ BCA assay kit (Thermo Fischer Scientific; Waltham, MA, USA). Protein samples were stored until future use at -80 °C.

2.4 Western Blots

Equal amounts of protein (20 μg) were loaded into 10% Mini-PROTEAN TGX precast gels (Bio-Rad; Hercules, CA, USA) and run for 100 minutes at 120 volts then transferred to a polyvinylidene fluoride (PVDF) blotting membrane. The membrane was blocked with 5% bovine serum albumin (BSA) in tris-buffered saline (TBS) with 0.01% Tween 20 (TBST) for 1 hour at room temperature then incubated overnight with primary antibody rabbit anti-sigma 1 receptor (Sig-1R; 1:500; Novus Biologicals, NBP1-82479), goat anti-5α-reductase (SRD5A; 1:500; Abcam,

ab110123), or mouse anti-3α-hydroxysteroid oxidoreductase (3α-HSOR; 1:500; Abcam, ab131375) at 4 ° C on a shaker. PVDF membranes were washed three times with TBST then incubated for one hour with the corresponding secondary antibody goat anti-mouse (1:8000), donkey anti-goat (1:8000), or goat anti-rabbit (1:8000) at room temperature on a shaker. PVDF membranes were washed three times with TBST and then visualized by Bio Rad ChemiDoc™ MP Imaging System. Protein was quantified by Image J (NIH) and normalized to β-actin or β3-tubulin. Tissue from the rat liver was used as a positive control for Sig-1R and 3α-hydroxysteroid oxidoreductase. Prostate tissue from adult male rats (n = 2) were used as a positive control for 5α-reductase. Tissue from adult male testis (n = 1) was used as a negative control for 5α-reductase. Liver was a positive control for 3α-HSOR and Sig-1R. Blocking peptide (NBP1-82479PEP, Novus Biologicals) was used to confirm specificity of the Sig-1R antibody.

2.5 Fluorescent Immunohistochemistry and Confocal Microscopy

Trigeminal ganglia were sectioned at 30 μm onto slides on a Leica cryostat, then stored at -20 °C. Tissue sections were fixed with 4% paraformaldehyde then preincubated with filtered normal goat or donkey serum in 0.1M phosphate buffered saline (PBS) with 0.1% Triton X-100 at room temperature for 90 minutes. Tissue sections were then incubated with primary antibody (see Table 1) rabbit anti-Sig-1R (1:100; Novus Biologicals, NBP1-82479), goat anti-SRD5A (1:100;

Abcam, ab110123), or mouse anti-3α-HSOR (1:100; Abcam, ab131375) overnight at room temperature. The tissue sections were then washed with 0.1M PBS three times and then incubated with the corresponding fluorescent-conjugated secondary antibody goat anti-rabbit Alexa-488 or 568 (1:300; Molecular Probes, Eugen, OR, USA), goat anti-mouse Alexa-488 or 568 (1:300; Molecular Probes), or donkey anti-goat Alexa-647 (1:300; Molecular Probes) for 90 minutes, rinsed, then subjected to a second round of primary immune-labeling overnight at room temperature with primary antibody for Isolectin IB4 (1:500; Fischer Scientific, I21414) in 0.1 mM $CaCl_2$, rabbit anti-TRPV1 (1:1000; Alomone, ACC-030), goat anti-Nav1.8 (1:1000; Alomone, SCN10A), goat anti-CGRP (1:1000; Abcam, AB36001) or rabbit anti-CGRP (1:1000; Immunostar 24112), guinea pig anti-PGP9.5 (1:1000; Millipore, AB5898), or chicken anti-NF200 (1:1000; Abcam, AB4680). Tissues were then rinsed with 0.1M PBS three times for 10 minutes each, incubated in the dark with fluorescent-conjugated secondary antibody goat anti-rabbit Alexa-568 (1:300; Molecular Probes), donkey anti-goat Alexa-488 or Alex-568 (1:300; Molecular Probes), or goat anti-chicken Alex-488 or Alexa-568 (1:300; Molecular Probes). Tissues incubated with isolectin IB4 primary antibody were first washed two times in 0.1M PBS then two times in 0.1 mM $CaCl_2$.

For secondary antibody incubation, tissues were incubated with fluorescent-conjugated secondary antibody for streptavidin (1:500; Molecular Probes) for 90 minutes at room temperature in the dark then slides were washed three times for

10 minutes with 0.1M PBS. Slides were then rinsed, dried, and cover slipped using Prolong Gold antifade mountant (P36930; Life Technologies). Mouse IgG2b isotype control (1:100; Novus Biologicals, NBP1-82479PEP) added to the slide instead of the primary antibody was used as a control for 3α-HSOR to account for any non-specific binding. Images were acquired with Nikon A1 Confocal Laser Microscope with NIS-Elements C software. All control images were obtained with the same gain settings as experimental images.

2.6 Data Analysis

Western blots were analyzed by densitometry using Image J software (National Institute of Health, Bethesda, MD). Each blot was selected as a region of interest, sampled 3 times, and analyzed for average gray scale pixel value (sum gray values/ number of pixels; 8-bit) following background correction (set at 150 pixels). Data were analyzed by one-way analysis of variance (ANOVA) in Graphpad Prism 8. Tukey's post hoc analysis was conducted. Statistical significance was tested at $p \leq 0.01$.

3 Results

Enzyme levels of 5α-reductase and 3α-HSOR and protein expression of Sig-1R were analyzed by western blot analysis (see Figure 1A, Figure 5A, and Figure 9A) across the female rat estrous cycle. Levels of 5α-reductase [$F(3,12) = 2.171$; $p > 0.05$;

see Figure 1B], 3α-HSOR [$F(3,12) = 0.3112$; $p > 0.05$; see Figure 5B], and Sig-1R [$F(3,12) = 1.036$; $p > 0.05$; see Figure 9B] did not significantly vary across the rat estrous cycle.

Immunostaining in the trigeminal ganglia indicated that 5α-reductase (see Figure 2C and Figure 2G) is present (see Figure 2D and Figure 2H) in neurons that were also immunoreactive for the transient receptor potential vanilloid 1 (TRPV1; see Figure 2A and Figure 2E) and in neurons that are immunoreactive for calcitonin gene-related peptide (CGRP; see Figure 2B and Figure 2F). Neurons that were immunoreactive for 5α-reductase (see Figure 3C and Figure 3G) were also immunoreactive (see Figure 3D and Figure 3H) for isolectin IB4 (see Figure 3A and Figure 3E) and protein gene product 9.5 (see Figure 3B and Figure 3F). Cells immunoreactive for 5α-reductase (see Figure 4C and Figure 4G) were also (see Figure 4D and Figure 4H) immunoreactive for sodium channel 1.8 (Nav1.8; see Figure 4A and Figure 4E) and neurofilament heavy 200 (NF200; see Figure 4B and Figure 4F). To account for background signal of the secondary antibody, tissues were stained the same, except the primary antibody was omitted.

Cells in the trigeminal ganglia that were immunoreactive for 3α-hydroxysteroid oxidoreductase (3α-HSOR; see Figure 6B and Figure 6F) were also immunoreactive (see Figure 6D and Figure 6H) for TRPV1 (see Figure 6A and Figure 6E) and CGRP (see Figure 6C and Figure 6G). Expression of 3α-HSOR (see Figure 7C

and Figure 7G) was present in the trigeminal ganglia. Specifically, 3α-HSOR was found in cells immunoreactive for isolectin IB4 (see Figure 7A and Figure 7E) and PGP9.5 (see Figure 7B and Figure 7F). 3α-HSOR (see Figure 8B and Figure 8F) was found in the trigeminal ganglia. Specifically, 3α-HSOR (see Figure 8D and Figure 8H) was present in cells immunoreactive for NF200 (see Figure 8A and Figure 8E) and Nav1.8 (see Figure 8C and Figure 8G). No staining was observed in slides where the primary antibody was omitted or when the slides were incubated with the isotype control instead of the primary antibody (data not shown).

The trigeminal ganglia were found to express Sig-1R (see Figure 10B and Figure 10F). Precisely, Sig-1R (see Figure 10D and Figure 10H) was present in cells immunoreactive for TRPV1 (see Figure 10A and Figure 10E) and CGRP (see Figure 10C and Figure 10G). Sig-1R were present in the trigeminal ganglia (see Figure 11B and Figure 11F). Specifically, Sig-1R (see Figure 11D and Figure 11H) were present in cells immunoreactive for isolectin IB4 (see Figure 11A and Figure 11E) and Nav1.8 (see Figure 11C and Figure 11G). Sig-1R (see Figure 12A and Figure 12D) was found in the trigeminal ganglia. Specifically, Sig-1R (see Figure 12C and Figure 12F) were present in cells immunoreactive for NF200 (see Figure 12B and Figure 12E). Sig-1R (see Figure 13A and Figure 13D) was found in the trigeminal ganglia. Precisely, Sig-1R (see Figure 13C and Figure 13) was present in cells

immunoreactive for PGP9.5 (see Figure 13B and Figure 13E). No staining was observed in slides where the primary antibody was omitted (data not shown).

Table 1.

Antibodies used for fluorescent immunohistochemistry

Primary Antibody	**Company**	**Cat #**	**Secondary Alexa Fluor**	**Target**
Goat anti-SRD5A1	Abcam	AB110123	Donkey anti-goat 647	5α-reductase
Mouse anti-AKR1C1/1C2	Abcam	AB131375	Goat anti-mouse 568 or Donkey anti-mouse 647	3α-hydroxysteroid oxidoreductase
Rabbit anti-Sigma-1R/OPRS1	Novus Biologicals	NBP1-82479	Donkey anti-rabbit 488 or 555 or Goat anti-rabbit 568	Sigma 1 receptor
Guinea pig anti-PGP9.5	Millipore	AB1774	Goat anti-guinea pig 568	Nerve fibers and neurons
Goat anti-Nav1.8	Alomone	SCN10A	Donkey anti-goat 647	Sodium channel 1.8; nociceptors
Goat anti-CGRP	Abcam	AB36001	Donkey anti-goat 488 or 568	Calcitonin gene-related peptide; peptidergic neurons
Rabbit anti-CGRP	Immunostar	24112	Goat anti-rabbit 568	Calcitonin gene-related peptide; peptidergic neurons
Guinea Pig anti-TRPV1	Neuromics	GP14100	Goat anti-rabbit 488	TRPV1 ion channels; nociceptor subtype
Isolectin IB4 biotin-XX conjugated	Fischer Scientific	I21414	Streptavidin 488 conjugate	Non-peptidergic neurons
Chicken anti-NF200	Abcam	AB4680	Goat anti-chicken 488 or 568	Neurofilament heavy; myelinated neurons

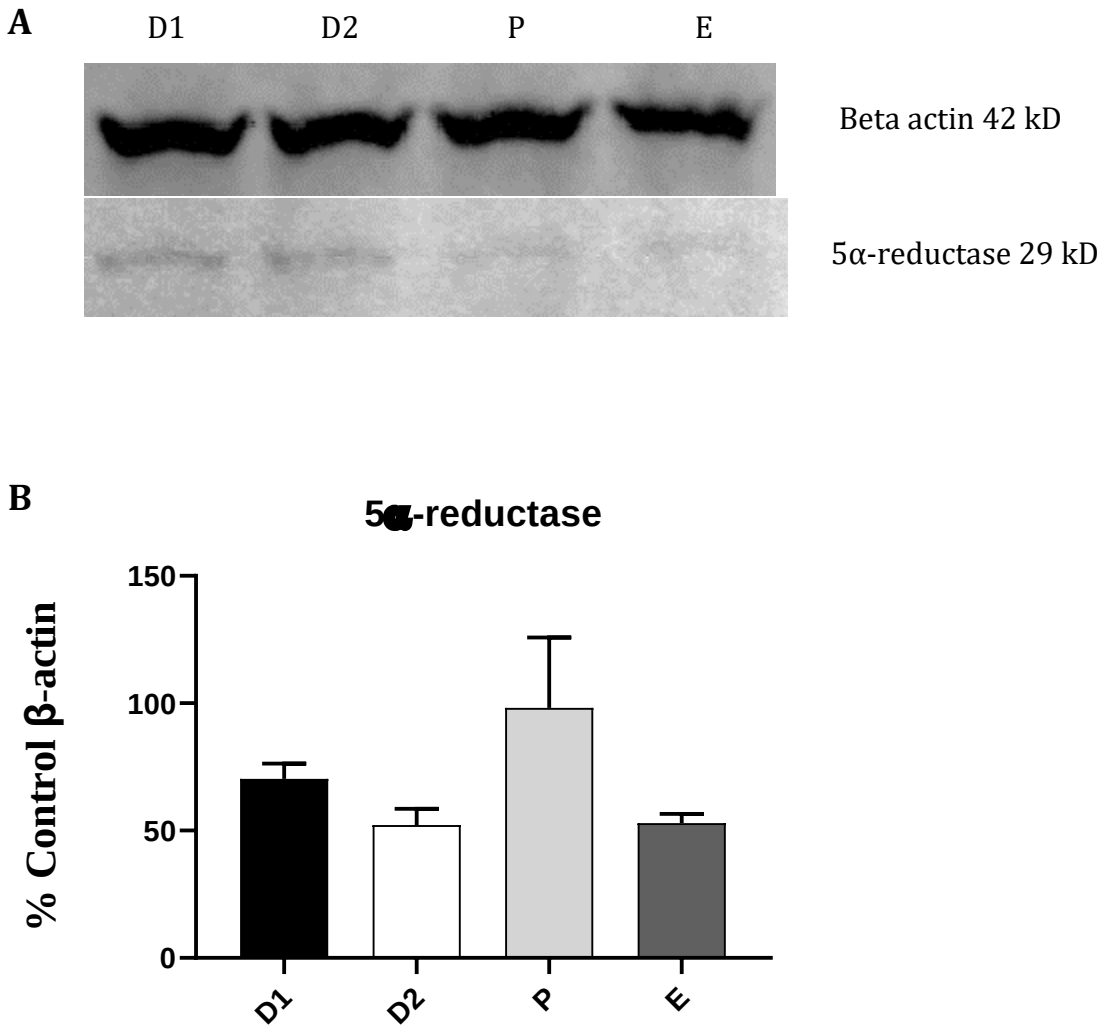

Figure 1. 5α-reductase expression in trigeminal ganglia does not vary across the estrous cycle of female rats.

5α-reductase protein expression done by western blot (**A**) and quantification (**B**) for each phase of estrous cycle [diestrus 1 (D1); diestrus 2 (D2); proestrus (P); estrus (E)] and quantification.

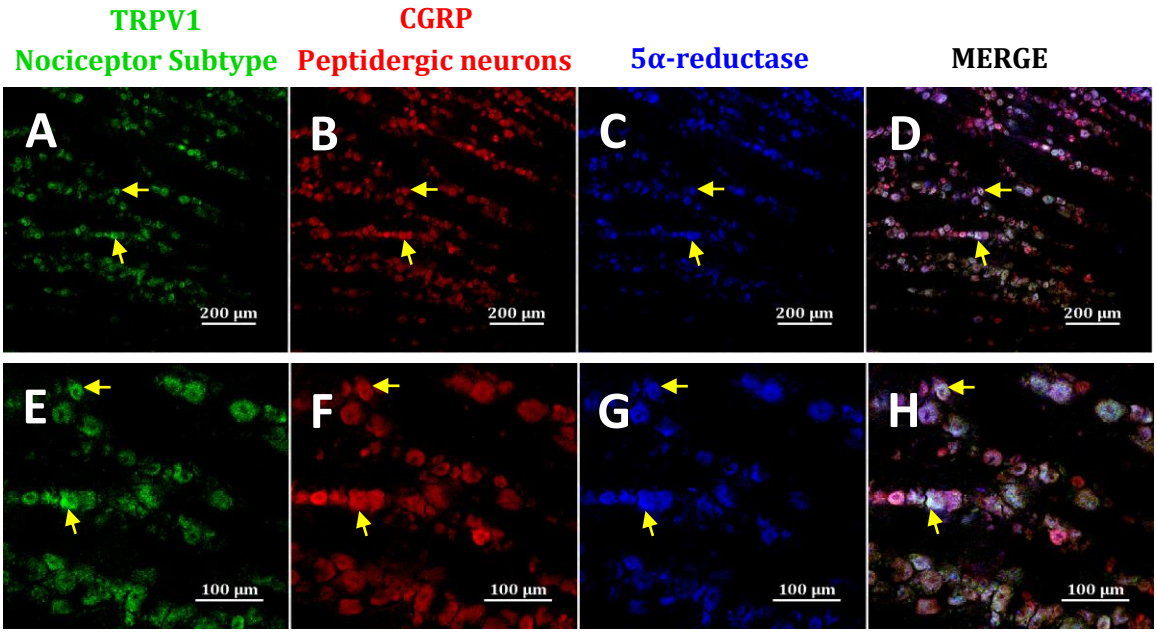

Figure 2. 5α-reductase expression with a subpopulation of nociceptors in trigeminal ganglia.

Immunofluorescent staining of transient receptor potential vanilloid 1 (TRPV1; subtype of nociceptor; green) (**A**) Calcitonin gene-related peptide (CGRP; peptidergic neurons; red) (**B**), 5α-reductase (blue) (**C**), and merged image (**D**) at 10X magnification. TRPV1 (green) (**E**), CGRP (red) (**F**), 5α-reductase (blue) (**G**), and merged image (**H**) at 20X magnification. Arrows indicate areas of coexpression.

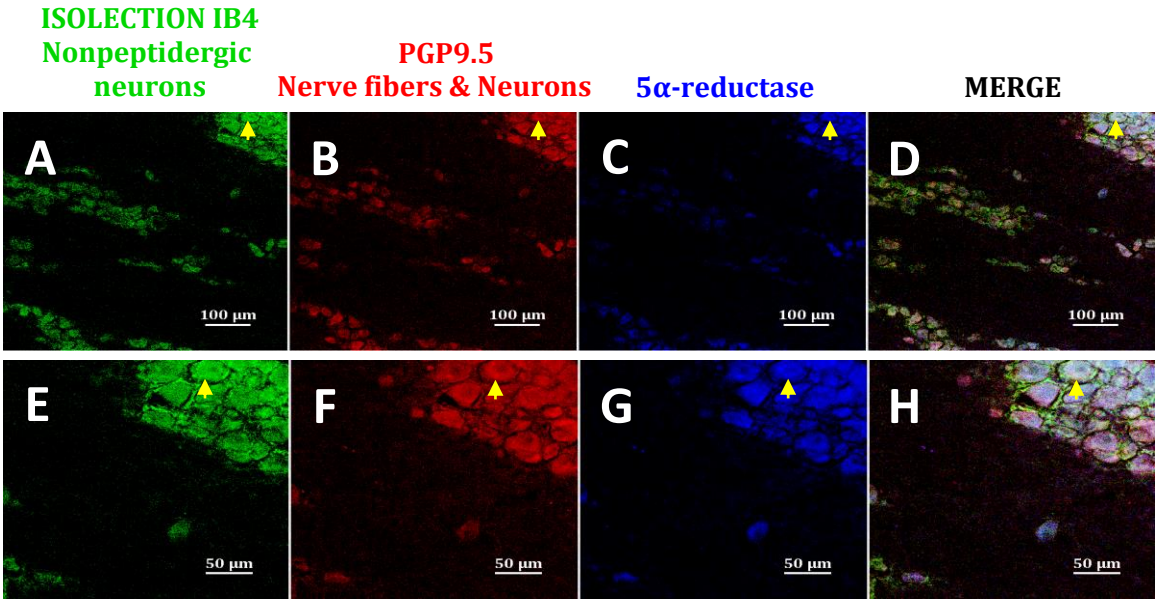

Figure 3. 5α-reductase expression in trigeminal ganglia with neurons, nerve fibers, and non-peptidergic neurons.

Immunofluorescent staining of isolectin IB4 (non-peptidergic neurons; green) (**A**), protein gene product 9.5 (PGP9.5; nerve fibers and neurons; red) (**B**), 5α-reductase (blue) (**C**), and merged image (**D**) at 10X. Isolectin IB4 (green) (**E**), PGP9.5 (red) (**F**), 5α-reductase (blue) (**G**), and merge images (**H**) at 20X magnification. Arrows indicate areas of coexpression.

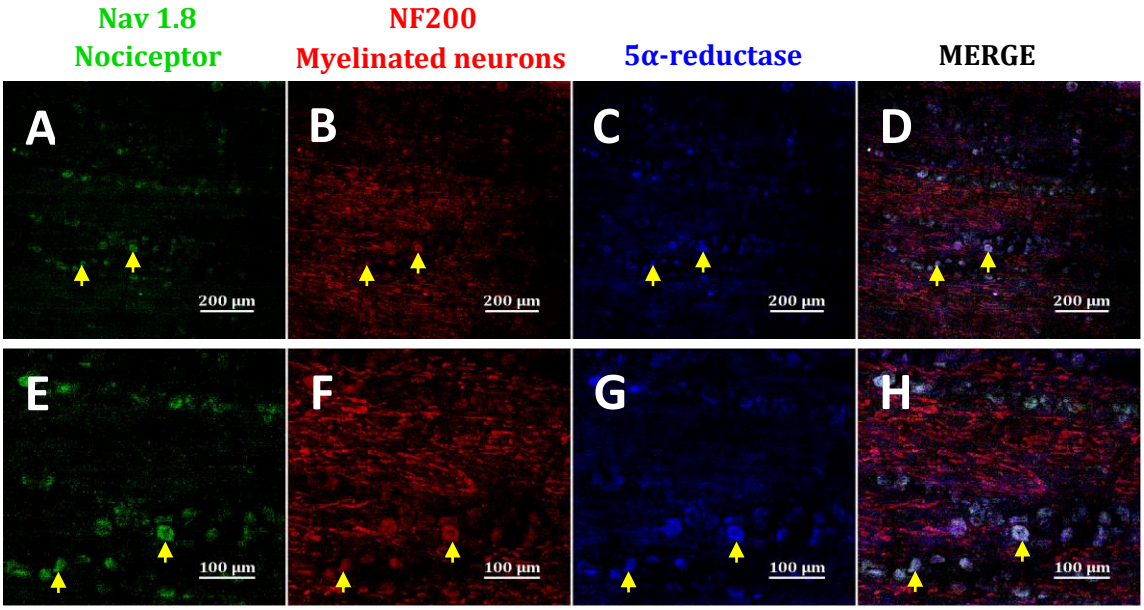

Figure 4. 5α-reductase expression in trigeminal ganglia with nociceptors and myelinated neurons.

Immunofluorescent staining of sodium channel 1.8 (Nav 1.8; nociceptor; green) (**A**), neurofilament heavy (NF200; myelinated neurons; red) (**B**), 5α-reductase (blue) (**C**), and merged image (**D**) at 10X magnification. Nav 1.8 (green) (**E**), NF200 (red) (**F**), 5α-reductase (blue) (**G**), and merged images (**H**) at 20X magnification. Arrows indicate areas of coexpression.

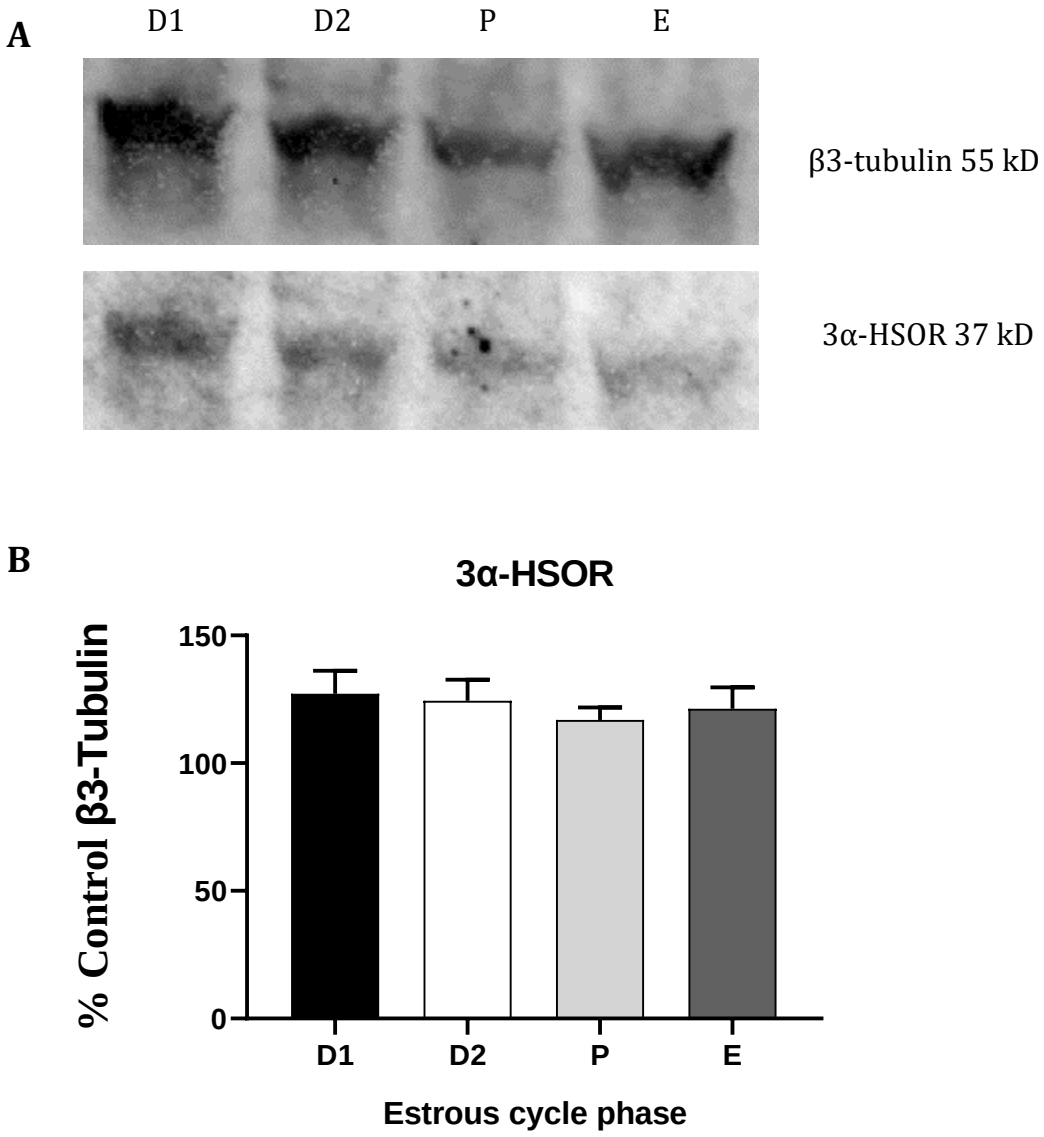

Figure 5. 3α-hydroxysteroid oxidoreductase (3α-HSOR) protein expression in trigeminal ganglia does not vary across the estrous cycle of female rats.

3α-HSOR protein expression done by western blot (**A**) and quantification (**B**) for each phase of estrous cycle [diestrus 1 (D1); diestrus 2 (D2); proestrus (P); estrus (E)].

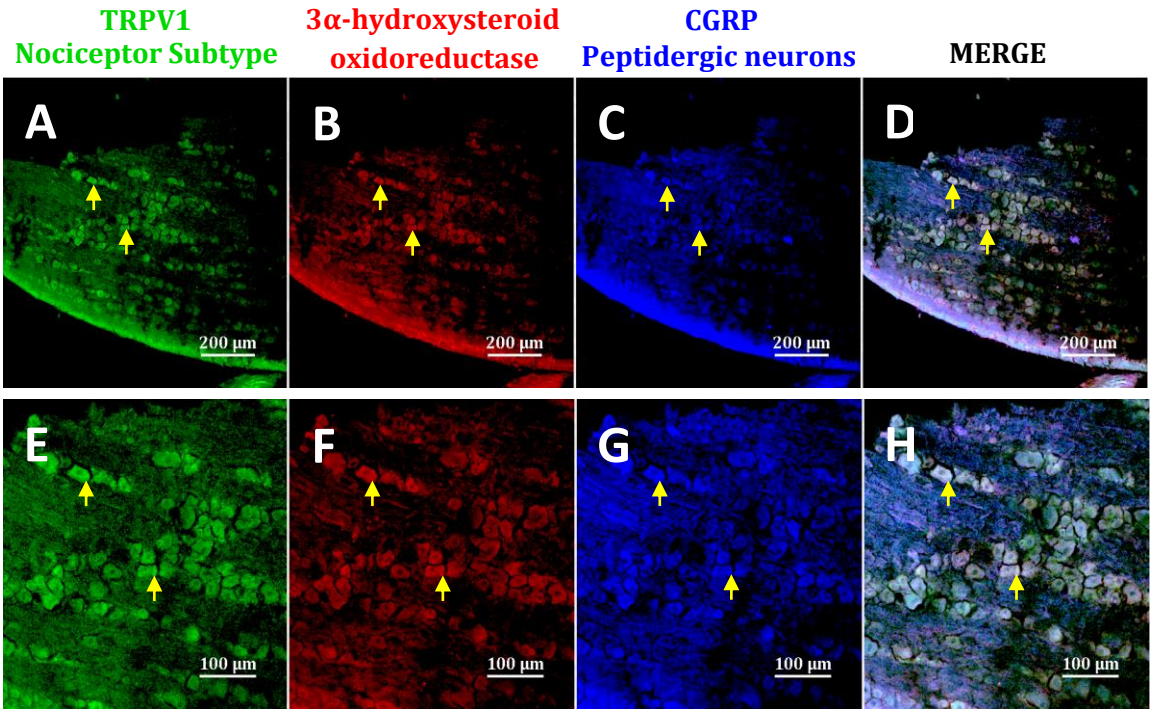

Figure 6. 3α-hydroxysteroid oxidoreductase expression in trigeminal ganglia with a subpopulation of nociceptors.

Immunofluorescent staining of transient receptor potential vanilloid 1 (TRPV1; subtype of nociceptor; green) (**A**), 3α-HSOR (red) (**B**), calcitonin gene-related peptide (CGRP; peptidergic neurons; blue) (**C**), and merged image (**D**) at 10X magnification. TRPV1 (green) (**E**), 3α-HSOR (red) (**F**), CGRP (blue) (**G**), and merged image (**H**) at 20X magnification. Arrows indicate areas of coexpression.

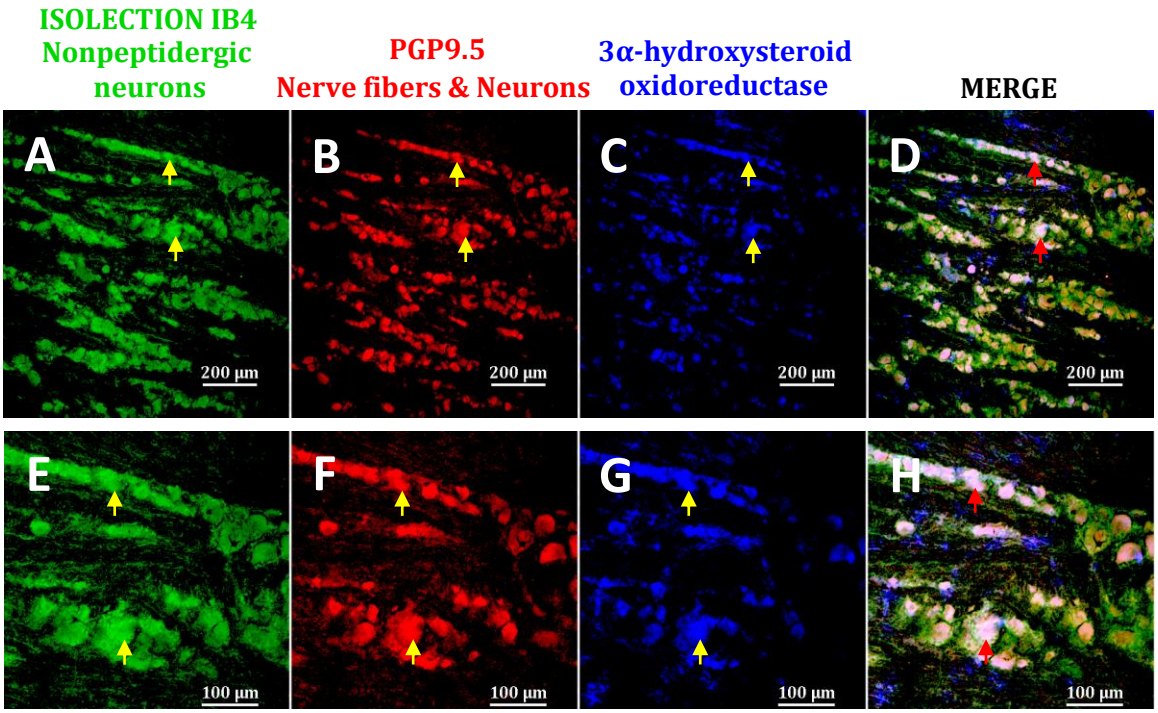

Figure 7. 3α-hydroxysteroid oxidoreductase expression in trigeminal ganglia with neurons, nerve fibers, and non-peptidergic neurons.

Immunofluorescent staining of isolectin IB4 (non-peptidergic neurons; green) (**A**), protein gene product 9.5 (PGP9.5; nerve fibers and neurons; red) (**B**), 3α-HSOR (blue) (**C**), and merged image (**D**) at 10X magnification. Isolectin IB4 (green) (**E**), PGP9.5 (red) (**F**), 3α-HSOR (blue) (**G**), and merge images (**H**)at 20X magnification. Arrows indicate areas of coexpression.

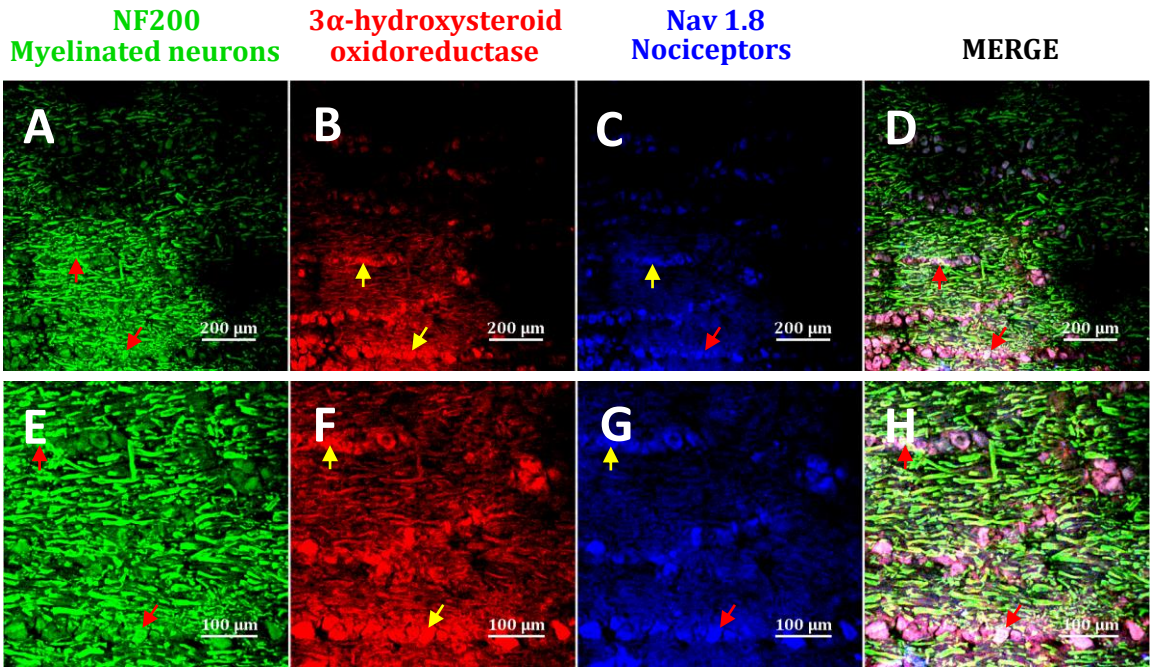

Figure 8. 3α-hydroxysteroid oxidoreductase expression in trigeminal ganglia with myelinated neurons and nociceptors.

Immunofluorescent staining of neurofilament heavy (NF200; myelinated neurons; green) (**A**), 3α-HSOR (red) (**B**), sodium channel 1.8 (Nav 1.8; nociceptor; blue) (**C**), and merged image (**D**) at 10X magnification. NF200 (green) (**E**), 3α-HSOR (red) (**F**), Nav 1.8 (blue) (**G**), and merged images (**H**) at 20X magnification. Arrows indicate areas of coexpression.

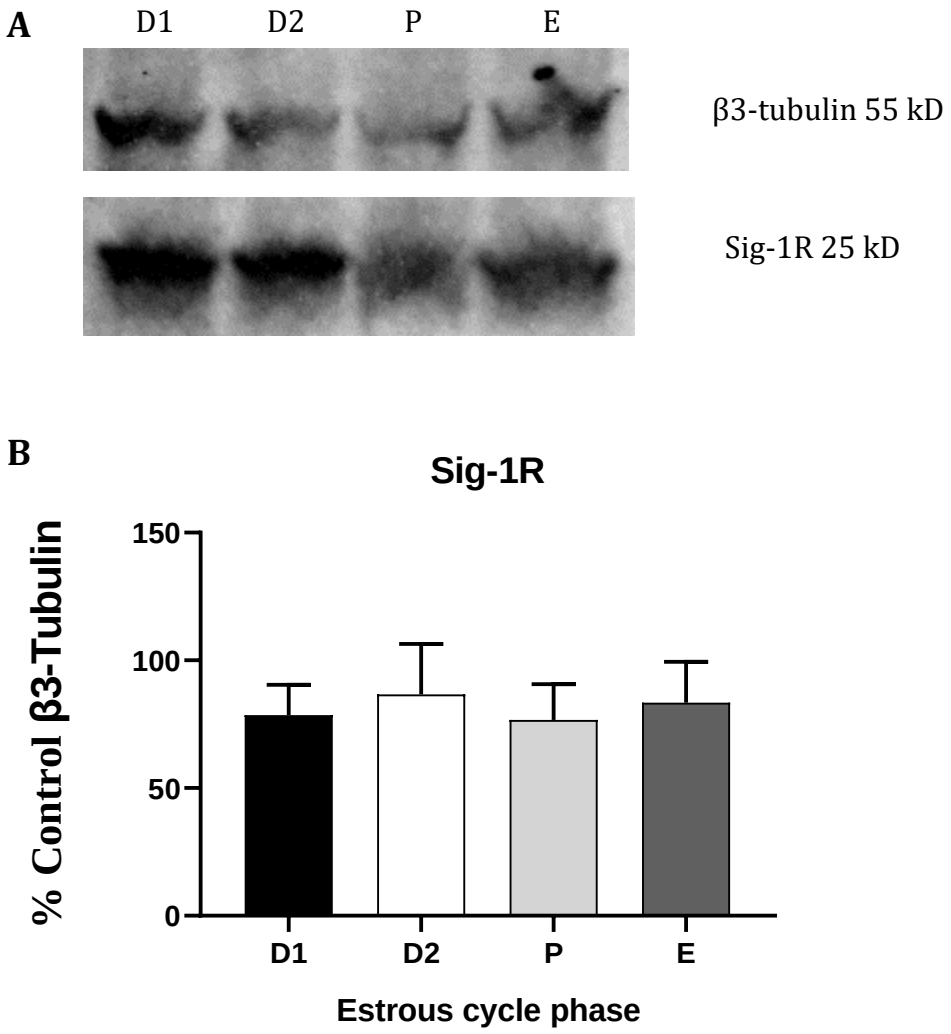

Figure 9. Sigma receptor (Sig-1R) expression in trigeminal ganglia does not vary across the estrous cycle of female rats.

Protein expression of sigma 1 receptor (Sig-1R) does not vary across the estrous cycle of female rats. Sig-1R protein expression done by western blot (**A**) and quantification (**B**) for each phase of estrous cycle [diestrus 1 (D1); diestrus 2 (D2); proestrus (P); estrus (E)].

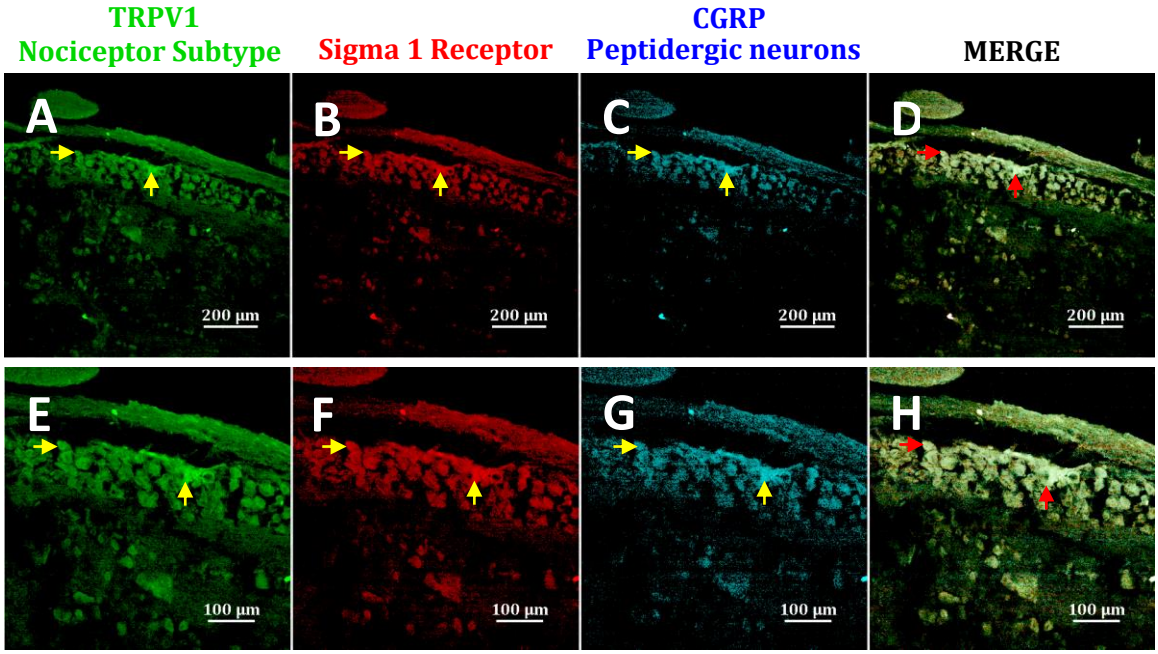

Figure 10. Sigma-1receptor expression in trigeminal ganglia with a subpopulation of nociceptors.

Immunofluorescent staining of transient receptor potential vanilloid 1 (TRPV1; subtype of nociceptor; green) (**A**), Sig-1R (red) (**B**), calcitonin gene-related peptide (CGRP; peptidergic neurons; cyan) (**C**), and merged image (**D**) at 10X magnification. TRPV1 (green) (**E**), Sig-1R (red) (**F**), CGRP (cyan) (**G**), and merged image (**H**) at 20X magnification. Arrows indicate areas of coexpression.

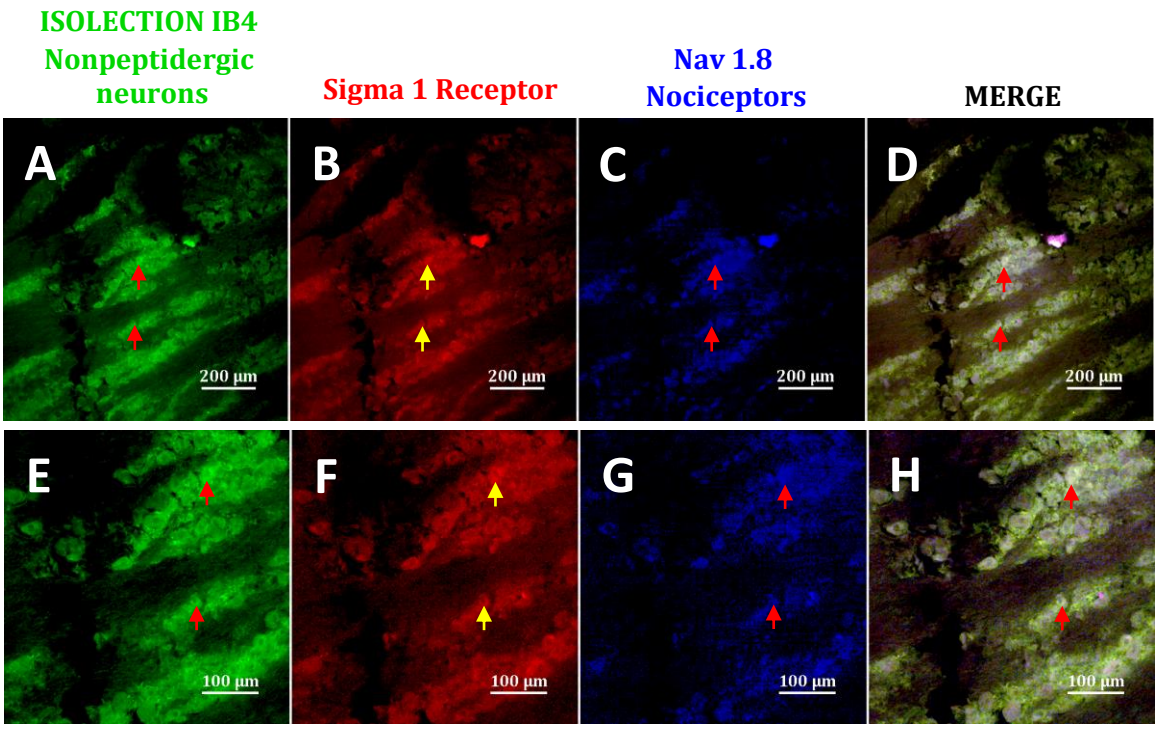

Figure 11. Sigma-1receptor expression in trigeminal ganglia with non-peptidergic neurons and nociceptors.

Immunofluorescent staining of isolectin IB4 (non-peptidergic neurons; green) (**A**), Sig-1R (red) (**B**), sodium channel 1.8 (Nav 1.8; nociceptor; blue) (**C**), and merged image (**D**) at 10X magnification. Isolectin IB4 (green) (**E**), Sig-1R (red) (**F**), Nav 1.8 (blue) (**G**), and merge images (**H**) at 20X magnification. Arrows indicate areas of coexpression.

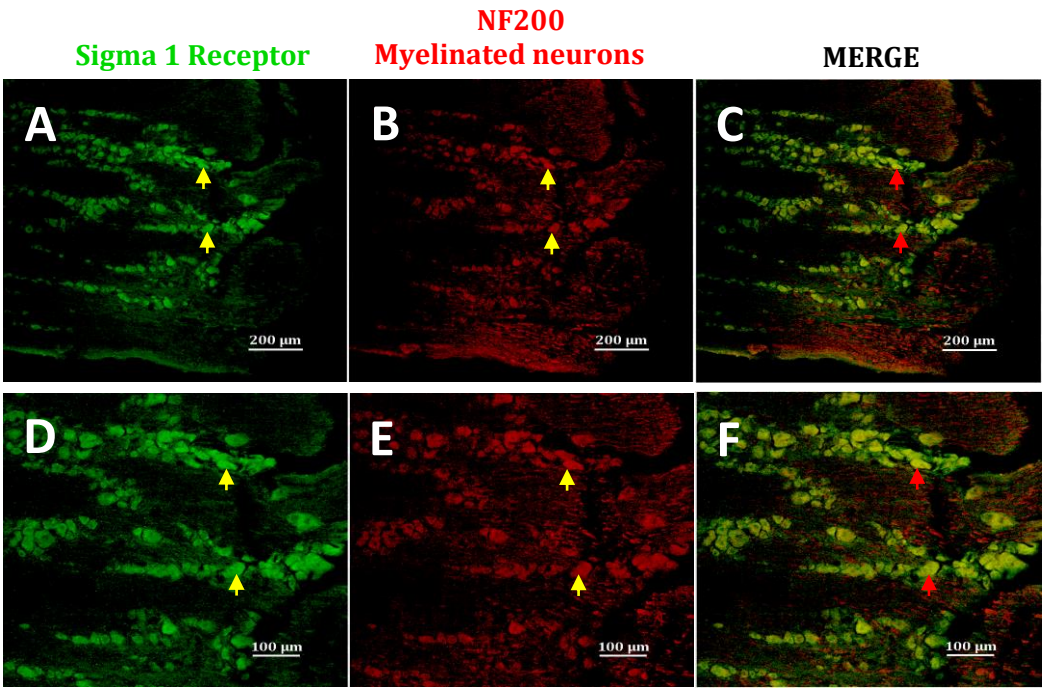

Figure 12. Sigma-1 receptor expression in trigeminal ganglia with myelinated neurons.

Immunofluorescent staining of Sig-1R (green) (**A**), neurofilament heavy (NF200; myelinated neurons; red) (**B**), and merged image (**C**) at 10X magnification. Sig-1R (green) (**D**), NF200 (red) (**E**), and merged image (**F**) at 20X. Arrows indicate areas of coexpression.

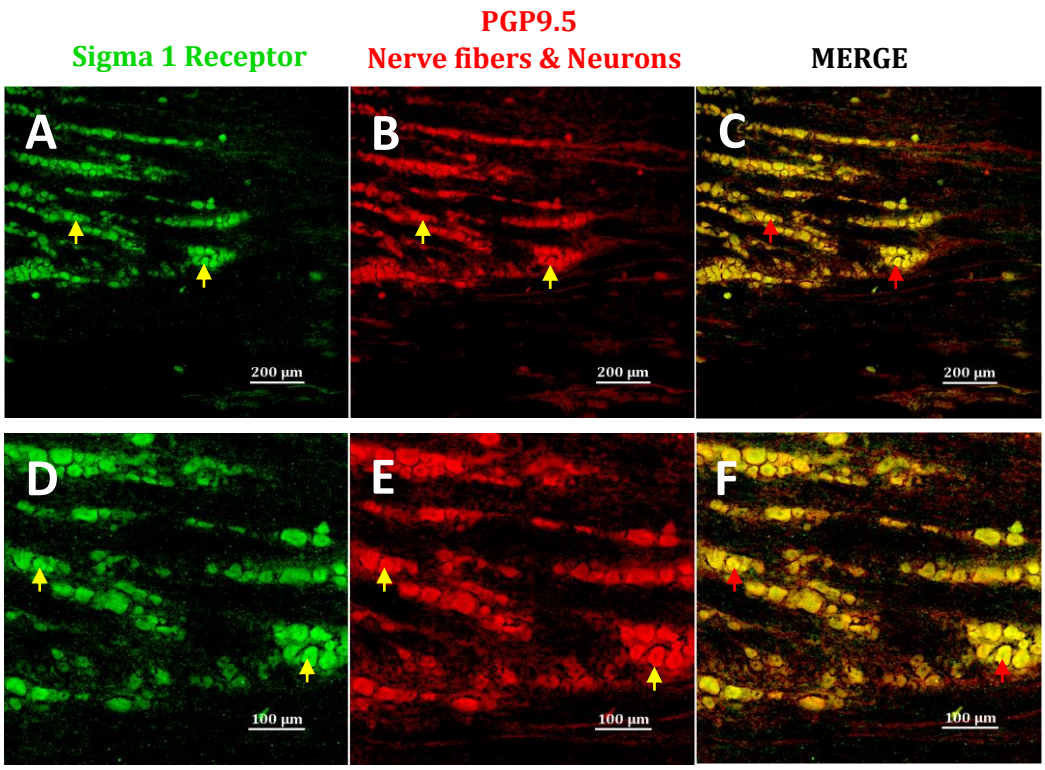

Figure 13. Sigma-1receptor expression in trigeminal ganglia with neurons and nerve fibers.

Immunofluorescent staining of Sig-1R (green) (**A**), protein gene product 9.5 (PGP9.5; nerve fibers and neurons; red) (**B**), and merged image (**C**) at 10X magnification. Sig-1R (green) (**D**), PGP9.5 (red) (**E**), and merged image (**F**) at 20X.

4 Discussion

The orofacial region is innervated by the trigeminal nerve, which has nociceptors that are sensitive to noxious chemical, mechanical, and thermal stimuli. Nociceptors innervating the cranio-orofacial region have cell bodies located in the trigeminal ganglia and are excited by noxious chemical, mechanical, and thermal stimuli. Excitation of the nociceptors is relayed to the trigeminal nucleus subcaudalis in the medullary spinal cord, which transmits the signal on to the thalamus and somatosensory cortex (Iwata, Takeda, Oh, & Shinoda, 2017). Progesterone's protective mechanisms can potentially occur within any of the peripheral and central nervous system anatomical locations mentioned above. Within the trigeminal system, estrogen upregulates inflammatory mediators (Kou et al., 2011; Puri, Bellinger, & Kramer, 2011; Yun, Chae, & Lee, 2008), ion channels (Bi et al., 2017; Hu et al., 2012; Wu et al., 2010; Wu, Hao, Kou, Gan, & Ma, 2015), and increases excitability of sensory neurons (Bereiter, Okamoto, & Bereiter, 2005; Flake, Bonebreak, & Gold, 2005; Okamoto, Bereiter, Thompson, Tashiro, & Bereiter, 2008). Although most studies focus on estrogen's effects on nociception, several have investigated progesterone's effects on the trigeminal system, Data from these studies indicates an antinociceptive role for progesterone within the trigeminal system (Fischer et al., 2008; Hornung, Benton, Tongkhuya, Uphouse, & Averitt, under revision; Xue et al., 2017).

Of the ovarian hormones, estrogen and progesterone, there is extensive evidence supporting a protective role for progesterone in nervous system diseases, disorders, and injuries. However, evidence is lacking for progesterone's neuroprotective effects for pain disorders that are more prevalent in women. Further, a potential anatomical substrate for progesterone's attenuation of orofacial pain is unknown. Here, we hypothesized that mechanisms for progesterone's neuroprotection would be expressed within the trigeminal ganglia making the trigeminal ganglia a potential therapeutic target for progesterone. We report that 5α-reductase. 3α-hydroxysteroid oxidoreductase, and the sigma 1 receptors (Sig-1R) are expressed in the trigeminal ganglia of naturally cycling female rats and protein levels do not vary with naturally fluctuating ovarian hormones.

Progesterone consistently downregulates these inflammatory mediators, thereby, reducing neuroinflammation (Hong et al., 2016; Hong, Liu, Zhang, Wu, & Hou, 2018; Litim, Morissette, & Di Paolo, 2017). Neuropeptides like calcitonin-gene related peptide (CGRP) are released following injury. CGRP is released in response to inflammation and as such, enhances nociception (Edvinsson, 2017). CGRP-positive neurons are expressed in the trigeminal ganglia of rats (Eftekhari et al., 2010; Eftekhari et al., 2015; Walker et al., 2015) and humans (Eftekhari et al., 2010). A deficiency in circulating progesterone levels augments CGRP in the periaqueductal gray (Wang et al., 2014). Progesterone could potentially augment CGRP within the trigeminal ganglia resulting in a reduction in pain sensitivity. Additionally, CGRP

activates transient receptor potential vanilloid 1 (TRPV1) receptors (Russell, King, Smillie, Kodji, & Brain, 2014) and TRPV1 receptors are highly expressed within the trigeminal ganglia (Diogenes et al., 2006). A decrease in CGRP release in the trigeminal ganglia from increased circulating progesterone may result in a cascade of events where TRPV1 receptor is not being activated, thus reducing pain sensitivity.

Additionally, the sigma 1 receptor is colocalized in the trigeminal ganglia with TRPV1 receptors (Ortiz-Renteria et al., 2018). Antagonism of the sigma 1 receptor by progesterone results in a downregulation of TRPV1 channel expression, which may be an antinociceptive mechanism of progesterone. Another neuropeptide, substance P, has been shown to inhibit progesterone metabolism within the spinal sensory circuit, thus resulting in a decrease in circulating allopregnanolone (Patte-Mensah, Kibaly, & Mensah-Nyagan, 2005). If substance P has the same effect in the trigeminal ganglia, then progesterone treatment may reverse this. Nevertheless, allopregnanolone is a positive allosteric modulator of the $GABA_A$ receptor, which is expressed in the trigeminal ganglia (Puri, Bellinger, & Kramer, 2011), therefore, allopregnanolone may enhance GABA-mediated antinociception.

The trigeminal ganglia are not the only potential anatomical substrate for progesterone's neuroprotective actions in orofacial pain. The trigeminal nucleus

caudalis in the medullary spinal cord is also a likely target. CGRP (Eftekhari & Edvinsson, 2011) and TRPV1 (Quartu et al., 2016) are both expressed in the trigeminal nucleus caudalis. Progesterone reduces CGRP accumulation within the trigeminal nucleus (Moussaoul, Duval, Lenoir, Garret, & Kerdelhue, 1996), which could lead to a reduction in pain. Progesterone may exert it antinociceptive properties through the sigma 1 receptor in the trigeminal nucleus caudalis. Chronic activation of the Sig-1R results in evoked nociception by activating the trigeminal nucleus caudalis (Pyun, Son, & Kwon, 2014). Additionally, progesterone's antinociceptive properties may result from reduction in TRPV1 expression, as observed in the trigeminal ganglia (Ortiz-Renteria et al., 2018) or just antagonizing the Sig-1R. Additionally, $GABA_A$ receptors are expressed in the trigeminal subnucleus caudalis (Castro et al., 2017) where allopregnanolone could enhance GABA-mediated antinociception. Further studies are warranted to determine mechanisms for progesterone's neuroprotection within the trigeminal nucleus.

Overall, we provide evidence that Sig-1Rs are available to bring progesterone in the trigeminal ganglia of female rats in similar concentrations across the estrous cycle and that progesterone could be metabolized locally in trigeminal sensory neurons as the required enzymes are present. The trigeminal ganglia are thus an anatomical substrate for the protective actions of progesterone and it metabolite allopregnanolone on orofacial or craniofacial pain. Future studies directly targeting progesterone's neuroprotective mechanisms within the trigeminal ganglia and

observing trigeminal pain behaviors are warranted by our neuroanatomical findings. Improving knowledge on progesterone's neuroprotection modes of action, especially for pain disorders that are more prevalent in women, may lead to more gender-specific therapeutic treatments and improvements in the quality of life for women in pain.

5 Conflict of Interest

The authors declare the research was conducted in the absence of any commercial, personal, or financial relationships that could inappropriately influence the research.

6 Author Contributions

RSH contributed to experimental design, conducting experiments, data analysis and interpretation, and preparation of the manuscript. NGR contributed to conducting experiments, data analysis and interpretation, and approval of the manuscript. DA contributed to experimental design, data analysis and interpretation, and preparation of the manuscript.

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CHAPTER V

SUMMARY/CONCLUSIONS

Progesterone was first classified as a female gonadal hormone for its role in female reproductive health. Throughout years of research, regulation of neuronal and glial cell functions, as well as, local progesterone synthesis by these cells in the nervous system have brought to light progesterone's role as a neurosteroid. Research indicates that progesterone plays a beneficial role in inflammation (Hong et al., 2016), edema (Kasturi & Stein, 2009), myelination and myelin repair (Labombarda et al., 2009), oxidative stress (Andrabi, Parvez, & Tabassum, 2017), excitotoxicity (Luoma, Kelley, & Mermelstein, 2011), neuronal loss (Jarahi, Sheibani, Safakhah, Torkmandi, & Rashidy-Pour, 2014), cognitive function (Cutler, Pettus, Hoffman, & Stein, 2005), and pain sensitivity (Dableh & Henry, 2011; Jarahi, Sheibani, Safakhah, Torkmandi, & Rashidy-Pour, 2014). The beneficial effects of progesterone have been evidenced by preclinical studies on traumatic brain injury, spinal cord injury, and stroke (Cervantes, Gonzalez-Vidal, Ruelas, Escobar, & Morali, 2002; Coronel, Labombarda, Villar, De Nicola, & Gonzalez, 2011; Pettus, Wright, Stein, & Hoffman, 2005). However, progesterone's effects on disorders, that are more prevalent in women than men, such as sexual dysfunction or pain, are understudied.

Most of the research on sexual function in women focuses on estrogen and progesterone is frequently overlooked. Both estrogen and progesterone protect against serotonin-induced sexual inhibition but only estrogen's mechanism is understood. Similarly, the effects of hormones on pain are becoming clearer, but progesterone is not typically a focus of pain research. This dissertation focused on the proposed therapeutic role that progesterone can have on both sexual receptivity and temporomandibular joint disorder (TMD) pain. We report the following main findings in this dissertation:

(i) Most of the research on sexual function in women focuses on estrogen and progesterone is frequently overlooked. Both estrogen and progesterone protect against serotonin-induced sexual inhibition but only estrogen's mechanism is understood. Similarly, the effects of hormones on pain are becoming clearer, but progesterone's attenuation of 5-HT_{1A}-induced sexual inhibition may involve the intracellular progesterone receptor,

(ii) ovariectomy reverses CFA-evoked orofacial mechanical allodynia,

(iii) orofacial mechanical allodynia reemerges following estrogen treatment,

(iv) the return of orofacial mechanical allodynia following estrogen treatment is attenuated rapidly with a dose of 16 mg/kg of progesterone,

(v) a lower dose of progesterone, 16 μg/kg but not 16 ng/kg, was sufficient in rapidly attenuating estrogen-exacerbated orofacial mechanical allodynia,

(vi) allopregnanolone also attenuated estrogen-exacerbated orofacial mechanical allodynia rapidly but only on the first day of treatment,

(vii) sigma 1 receptors (Sig-1Rs) are expressed on nociceptive sensory neurons of the trigeminal ganglia of female rats and levels do not vary across the rat estrous cycle, and

(viii) the enzymes 3α-hydroxysteroid oxidoreductase and 5α-reductase are also expressed in nociceptive sensory neurons of the female rat trigeminal ganglia indicating that progesterone metabolism can occur in pain-sensing neurons.

To understand the mechanisms that result in human female sexual dysfunction induced by the antidepressants SSRIs, researchers utilize rodent models that display altered sexual behavior in response to SSRI injections. Female rodent sexual behavior consists of attractive, proceptive, and receptive behaviors (Beach, 1976). Of these, proceptive and receptive behaviors are the most studied in female rodents. Proceptive behaviors (ear wiggling and hopping and darting) are exhibited by the female to entice the male to mate with her (Bergheim, Chu, & Agmo, 2015; Micevych, Soma, & Sinchak, 2008). Lordosis (degree of arching of the spine and dorsoflexion of the tail) is a receptive behavior displayed by the female to facilitate

ejaculation by the male (Bergheim, Chu, & Agmo, 2015; Micevych, Soma, & Sinchak, 2008). Estrogen is more important for the emergence of rodent sexual behavior and lordosis, but progesterone allows for the expression of the entire repertoire of behaviors and is required for proceptive behaviors. Animal models of lordosis have been utilized to study the effects of SSRIs, such as fluoxetine, on sexual behavior and treatment with fluoxetine results in inhibition of lordosis (Adams, Heckard, Hassell, & Uphouse, 2012; Frye & Rhodes, 2010; Matuszczyk, Larsson, & Eriksson, 1998; Miryala et al., 2011).

Of serotonin's many receptors, the 5-HT_{1A} receptor has been implicated in SSRI-inhibition of sexual behavior because fluoxetine's inhibition of sexual behavior is reversed with a 5-HT_{1A} receptor antagonist (Guptarak, Sarkar, Hiegel, & Uphouse, 2010). In support, 5-HT_{1A} receptor agonism consistently inhibits sexual behavior (Kishitake & Yamanouchi, 2005; Selvamani, Lincoln, & Uphouse, 2007; Uphouse & Wolf, 2004), but is less effective when estrogen and progesterone are onboard (Jackson & Uphouse, 1996; Trevino, Wolf, Jackson, Price, & Uphouse, 1999; Truitt et al., 2003). Estrogen reduces 5-HT_{1A} receptor agonism through uncoupling the receptor from its inhibitory G protein (Mize & Alper, 2002), but is it not known how progesterone alters 5-HT_{1A} receptor agonism. Our data indicates that progesterone's attenuation of 5-HT_{1A} function on sexual behavior involves the intracellular progesterone receptor (iPR). Progesterone either reduces 5-HT_{1A} receptor binding, reduces 5-HT_{1A} receptor expression, or disrupts 5-HT_{1A} receptor

coupling to intracellular signaling cascades. Clinical studies report a negative correlation between serum progesterone levels and 5-HT_{1A} receptor binding (Lanzenberger et al., 2011; Stein et al., 2014) and preclinical studies report that progesterone reduces 5-HT_{1A} expression in the brain of macaques (Henderson & Bethea, 2008). Alternatively, progesterone may increase phosphorylation of the 5-HT_{1A} receptor, thereby, causing it to uncouple from its inhibitory G protein, similar to estrogen (Mize & Alper, 2002). Since compounds that increase cAMP facilitate rodent sexual behavior and attenuate the inhibition of sexual behavior induced by 5-HT_{1A} receptor activation (Jackson & Uphouse, 1998; Uphouse, Maswood, & Jackson, 2000), and cAMP (cyclic adenosine monophosphate) levels increase 2 to 4 hours after progesterone treatment (Collado, Rodriguez-Manzo, & Cruz, 1985), progesterone through actions of the iPR may affect 5-HT_{1A} activation on sexual behavior by increasing cAMP. While our data indicate progesterone is working via the iPR receptor, actions at mPRs cannot be ruled out. Further studies would need to be conducted to determine what role, if any, the mPRs play a role in progesterone's attenuation of sexual inhibition induced by 5-HT_{1A} receptor activation.

Clinical studies on women with sexual dysfunction have reported improvements with hormone supplementation with either an estrogen, a progestin, or a combination of both (Caruso et al., 2017; Gonzalez, Viafara, Caba, & Molina, 2004; Guida et al., 2019; Skrzypulec & Drosdzol, 2008; Ulubay et al., 2017). It is not

known if progesterone administration would reduce the sexual side effects in women taking SSRIs, what dose would be effective, or how long before progesterone's protective effects are evident. The data suggest that a progestin taken before an SSRI may attenuate the negative effects on female sexual function. In support, clinical studies report an intrauterine contraceptive device containing only a progestin, which releases 20 μg daily, improves sexual function (Arlier et al., 2017; Ulubay et al., 2017) and reduces menstrual pain (Ji et al., 2014; Ulubay et al., 2017). Together, these data suggest a low dose of progestin each day may be adequate for improving serotonin-induced sexual dysfunction. Of note, clinical studies report an increased risk of breast cancer, pulmonary embolism, stroke, and coronary heart disease with progestins (Brinton et al., 2008; Chlebowski et al., 2015; Heiss et al., 2008; Rossouw et al., 2002). However, these increased risks have been associated with synthetic progestins (Hulley et al., 2002; Pike & Ross, 2000; Santen, Pinkerton, McCartney, & Petroni, 2001; Stahlberg, Pederson, Lynge, & Ottesen, 2003) and not natural progestins (Fournier, Berrino, Riboli, Avenel, & Clavel-Chapelon, 2005).

While progesterone attenuates the negative side effects of 5-HT_{1A} receptor activation on sexual receptivity, progesterone also attenuates pain sensitivity. Women with sexual dysfunction that experience pain during intercourse, report a significant improvement while on HRT (Gonzalez, Viafara, Caba, & Molina, 2004).

Beyond pain associated with sexual dysfunction, women are more sensitive and less tolerant to pain than men (Fillingim, King, Ribeiro-Dasilva, Rahim-Williams, & Riley, 2009; Rosseland & Stubhaug, 2004) and pain sensitivity varies across the menstrual cycle (Hellstrom & Anderberg, 2003; LeResche, Mancl, Sherman, Gandara, & Dworkin, 2003; Sherman & LeResche, 2006). Further, a variety of pain conditions more prevalent in women and some, including TMD, have been strongly linked to modulation by ovarian hormones (Riley, Robinson, Wise, & Price, 1999; Sherman & LeResche, 2006) and HRT (LeResche, Saunders, Von Korff, Barlow, & Dworkin, 1997; Wise, Riley, & Robinson, 2000).

Preclinical TMD models mainly focus on estrogen's modulation of pain, while progesterone's role is often overlooked. However, data from neuropathic pain models report that progesterone is anti-inflammatory (Lei et al., 2014; Preciado-Martinez et al., 2018) and antinociceptive (Kim et al., 2012; Liu et al., 2014). Data is even more limited on progesterone's antinociceptive effects on trigeminal pain, including TMD. Progesterone has been reported to improve neuropathic pain associated with demyelination of the trigeminal nerve root (Kim et al., 2012). Interestingly, daily administration of progesterone prior to inflammation attenuated TMJ nociception and inflammation (Xue et al., 2017), however this study design does not properly model clinical presentation of postmenopausal women on HRT. Our study is the first to report that ovariectomy reverses mechanical allodynia at the rat TMJ. These results model postmenopausal women reporting that TMD pain

dissipates following menopause. Importantly, we found that mechanical allodynia in the TMJ reemerges following estrogen treatment, which models postmenopausal women who report that TMD pain reemerges following estrogen replacement therapy (LeResche, Saunders, Von Korff, Barlow, & Dworkin, 1997). We also report that acute and continuous administration of progesterone, and acute (but not continuous) allopregnanolone, were able to rapidly reduce the estrogen-evoked mechanical allodynia at the TMJ.

The rapid effects of progesterone may involve progesterone acting at either iPRs, mPRs, or Sig-1Rs. As progesterone, but not allopregnanolone, was both fast and long-acting, it is likely that progesterone is displaying anti-inflammatory properties to reduce TMD pain. This is supported by reports that progesterone can act at the iPR to alter gene expression of and downregulate inflammatory mediators (Coronel, Labombarda, De Nicola, & Gonzalez, 2014; Coronel et al., 2016; Xue et al., 2017), some of which are upregulated by estrogen (Kou et al., 2011). Downregulation of the NF-kB pathway by progesterone treatment is partially reversed with an iPR antagonist indicating progesterone's actions on the NF-kB pathway involve more than just the iPR (Xue et al., 2017). Further, downregulation of inflammatory cytokines by progesterone treatment is blocked with pretreatment by an iPR antagonist, indicating gene modulation of cytokines by progesterone requires the iPR (Lei et al., 2014). Genomic effects of progesterone on inflammatory mediators are reported at 3, 6, 24, and 48 hours post-progesterone treatment (He,

Evans, Hoffman, Oyesiku, & Stein, 2004; Sarkaki, Khaksari Haddad, Soltani, Shahrokhi, & Mahmoodi, 2013; Wang et al., 2011). Additionally, pain-modulating regions in the rat central nervous system, including the thalamus, medulla, and spinal cord, express iPR mRNA (Pang, Dong, & Thomas, 2013). Taken together, these data suggest the iPR plays a role in the rapid and long-lasting attenuation of orofacial mechanical allodynia by progesterone observed in the current study. Progesterone (and allopregnanolone) also binds mPRs, which are coupled to either a stimulatory G protein that increases cAMP (Pang, Dong, & Thomas, 2013) or an inhibitory G protein that reduces cAMP (Smith et al., 2008). While mRNA for these receptors are expressed within regions associated with pain modulation (Pang, Dong, & Thomas, 2013) and in the trigeminal ganglia (Manteniotis et al., 2013), whether they play a role in pain modulation has not been reported. It remains possible that progesterone and/or allopregnanolone's rapid effects in the current study involve mPRs since inhibiting adenylyl cyclase (Cheng et al., 2019) or cAMP (Shao et al., 2016) has analgesic and antinociceptive effects, however a lack of commercially available pharmacologics limits exploration of this possibility.

Alternatively, progesterone's rapid effects on orofacial pain may involve the Sig-1R. The Sig-1R is a non-opioid chaperone receptor located in the plasma membrane of the endoplasmic reticulum (Hayashi & Su, 2003) and is expressed in brain regions associated with pain modulation (Alonso et al., 2000; Sanchez-Fernandez et al., 2014). Here we show Sig-1R expression also includes the

trigeminal ganglia of naturally, cycling female rats. Sig-1R agonists in the presence of injury or inflammation, enhance nociceptive responses as rapidly as 5 minutes following formalin injection (Cendan, Pujalte, Portillo-Salido, & Baeyens, 2005), 10 minutes after NMDA-induced nociception (Kim et al., 2008b), or 3 hours after carrageenan-induced inflammation (Parenti et al., 2014). Progesterone, which is a potent Sig-1R antagonist (Johannessen, Fontanilla, Mavlyutov, Ruoho, & Jackson, 2011), has been shown to have antinociceptive properties through actions at the Sig-1R (Ueda et al., 2001). The rapid antinociceptive effects Sig-1R antagonists have on nociceptive responses, along with our data showing rapid antinociceptive effects of progesterone, suggest a potential role for Sig-1R in progesterone's attenuation of the return of mechanical allodynia. More research is needed to better understand the potential role Sig-1R may play progesterone's rapid attenuation of orofacial pain.

Antinociceptive effects of allopregnanolone have been shown in models of inflammatory pain (Ocvirk, Pearson Murphy, Franklin, & Abbott, 2008), diabetic neuropathy (Afrazi & Esmaeili-Mahani, 2014), and postoperative pain (Fujita, Fukuda, Sato, Takasusuki, & Tanaka, 2018). Allopregnanolone biosynthesis involves two enzymes, 5α-reductase and 3α-hydroxysteroid oxidoreductase. Here, we show both enzymes are expressed within the trigeminal ganglia of naturally, cycling female rats. Allopregnanolone does not bind to the iPR or the Sig-1R, but can act at mPRs and is known to reduce pain via allosteric modulation of the $GABA_A$ receptor

(for review, see (Wang, 2011). $GABA_A$ receptors are expressed in the trigeminal ganglia (Puri, Bellinger, & Kramer, 2011), spinal tract nucleus (Kondo et al., 1995), spinal cord (Bohlhalter, Weinmann, Mohler, & Fritschy, 1996), and thalamus (Jia, Pignataro, & Harrison, 2007). In addition, endogenous levels of allopregnanolone and the enzyme that synthesizes allopregnanolone from 5α-dihydroprogesterone, 3α-hydroxysteroid oxidoreductase, are increased in the brain and/or spinal cord of rats with spinal cord injury (Kawano et al., 2011; Meyer, Venard, Schaeffer, Patte-Mensah, & Mensah-Nyagan, 2008). Since nociception increases the biosynthesis of allopregnanolone, the additional allopregnanolone administration performed in our study could trigger downregulation of $GABA_A$ receptors (Turkmen, Backstrom, Wahlstrom, Andreen, & Johansson, 2011), which may account for the loss of analgesia with repeated administration in our study. Taken altogether, we postulate that the rapid, acute effects of progesterone in the current study involve these multiple mechanisms: (i) progesterone or allopregnanolone binds to mPR leading to a decrease in cAMP production, (ii) progesterone binding to iPR reduces inflammatory mediator production, (iii) progesterone antagonizes Sig-1R in trigeminal sensory neurons to rapidly inhibit pain, and (iv) progesterone is metabolized to allopregnanolone in the trigeminal sensory neurons to inhibit pain via $GABA_A$ receptors.

We have also provided evidence that our preclinical data corroborate clinical reports in women with sexual dysfunction and TMD pain. Clinical studies

investigating pain thresholds report that women using hormonal contraceptives with a continuous release of a progestin had higher pain thresholds than women not using hormonal contraceptives (Maximo et al., 2015). The majority of women with either endometriosis-associated pelvic pain (Vercellini et al., 2018) or mastodynia (Winkler, Schindler, Brinkmann, Ebert, & Oberhoff, 2001) report improvement in pain while on a low dose progestin monotherapy. Dosage and duration are critical components to consider as 16 mg/kg of progesterone is the optimal dose in improving cognitive function (Goss, Hoffman, & Stein, 2003), while the efficacy is lost at suboptimal doses (Goss, Hoffman, & Stein, 2003; Murphy, Traystman, Hurn, & Duckles, 2000). On the other hand, efficacy of progesterone can be lost at high dosages as well (Wali, Ishrat, Won, Stein, & Sayeed, 2014; Yousef, Balzer, Crago, Poloyac, & Sherwood, 2014). Duration is just as important. Daily progesterone administration for five days provided greater symptom improvement versus three days of treatment (Galani, Hoffman, & Stein, 2001; Shear, Galani, Hoffman, & Stein, 2002). Further, preclinical doses and duration are not equivalent to requirements for humans. ProTECT, a phase III clinical study for patients with traumatic brain injury, conducted a study using 12 mg/kg/day of progesterone for three days without significant improvement (Wright et al., 2014) despite the patient's serum progesterone levels being over three times greater (Wright, Ritchie, Mullins, Kellermann, & Denson, 2005) than levels reported in rats (Wright, Bauer, Hoffman, & Stein, 2001).

In conclusion, the important role of progesterone in female prevalent sexual and orofacial pain disorders opens the door for gender-based therapies. Progesterone protects against serotonin-induced sexual dysfunction in rats, suggesting women experiencing serotonin-induced sexual dysfunction may benefit from a multimodal therapy that includes a low dose of progesterone. Multiple benefits are experienced by a daily, low dose of progesterone as sexual function and menstrual cramps are improved (Ulubay et al., 2017). Since progesterone attenuates menstrual cramps, post-menopausal women experiencing a reemergence of their TMD pain following estrogen therapy, may reap the benefits as well. For these women, addition of a low dose of progesterone to their estrogen therapy may help alleviate the return of TMD pain. Progesterone's beneficial effects involve multiple mechanisms providing acute and long-lasting protective effects. Gender-based treatments for women with disorders that are highly prevalent in women could include a multimodal treatment approach that involves progesterone.

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