

Original Research

Estrogen exacerbates the nociceptive effects of peripheral serotonin on rat trigeminal sensory neurons

Sukhbir Kaur, Hanna McDonald, Sirima Tongkhuya, Cierra M.C. Lopez, Sushmitha Ananth, Taylor M. Hickman, Dayna L. Averitt*

Department of Biology, Texas Woman's University, Denton, TX 76204, United States

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ABSTRACT

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

Introduction

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

pain is often relieved during pregnancy and menopause (Fejes-Szabo et al., 2018; Marcondes et al., 2002). These clinical reports implicate a modulatory role of gonadal hormones on the sensory system innervating the orofacial region.

While the sensory neurons of the dorsal root ganglia (DRG) innervate the trunk and extremities, the sensory neurons of the trigeminal ganglia (TG) innervate the cranial and orofacial tissues. A subpopulation of trigeminal neurons expresses the Transient Receptor Potential Vanilloid 1 (TRPV1) ion channel, a nociceptor activated by diverse noxious stimuli, including noxious heat (>42 °C), capsaicin, protons, and endogenous lipids; resulting in calcium influx in the cell and release of calcitonin gene related peptide (CGRP), thus initiating nociceptive signaling (Basbaum et al., 2009; Jeske et al., 2008; Ruparel et al., 2012). Activation and sensitization of trigeminal nociceptors underlying orofacial pain involves recruitment of immune cells such as mast cells, platelets, and macrophages (Ji et al., 2016; Shinoda et al., 2019). These immune cells release an acidic milieu of proinflammatory and pronociceptive mediators, such as bradykinin, histamine, prostaglandins, and

* Corresponding author at: Department of Biology, Texas Woman's University, 304 Administration Drive, Denton, TX 76204, United States.

E-mail addresses: slulla@twu.edu (S. Kaur), HannaMcDonald@my.unthsc.edu (H. McDonald), tongkhuya@livemail.uthscsa.edu (S. Tongkhuya), Cierra.lopez@tempus.com (C.M.C. Lopez), thickman1@twu.edu (T.M. Hickman), daveritt@twu.edu (D.L. Averitt).

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serotonin (5HT). These mediators activate signaling cascades in sensory neurons to contribute to peripheral sensitization, thus reducing the threshold for activation of nociceptors which underlies an increase in pain sensitivity and may ultimately contribute to the development of chronic pain conditions.

In the periphery, 5HT is stored and produced by injured epithelial cells, gut enterochromaffin cells, macrophages, T cells, mast cells, and platelets (Herr et al., 2017; Mössner and Lesch, 1998; Ni et al., 2008; Spohn and Mawe, 2017). 5HT is a major pro-inflammatory molecule that can act through seven known subtypes of 5HT receptors (5HT₁-5HT₇) to induce pain. Previous studies in male rats have shown that excitatory 5HT_{2A} and 5HT₃ receptor subtypes are localized to trigeminal sensory neurons that also express TRPV1, leading to an enhanced capsaicin-evoked Ca⁺² influx and CGRP release, that is attenuated in presence of 5HT_{2A} and 5HT₃ receptor antagonists (Loyd et al., 2012a; Loyd et al., 2011). 5HT also potentiates capsaicin-evoked CGRP release in human dental pulp isolated from females during the luteal phase of the menstrual cycle (Loyd et al., 2012b). Similarly, 5HT injection into the masseter muscle of healthy human females leads to increased hyperalgesia and allodynia, which is abolished in presence of 5HT₃ antagonist, granisetron (Ernberg et al., 2000). In support, our lab has reported that intraplantar injection of 5HT evokes greater and longer lasting pain behaviors in female rats during proestrus and estrus compared to males, diestrus, and ovariectomized females and these pain behaviors are attenuated in the presence of the 5HT_{2A} antagonist, M100907 (Kaur et al., 2018). We have also reported that blocking the 5HT₃ receptor with granisetron does not attenuate 5HT-evoked orofacial pain behaviors in male and female rats (Kaur et al., 2021a). This suggests a potential neuromodulatory role of 5HT_{2A} receptors in sex differences in trigeminal pain processing.

While progesterone plays a clear antinociceptive and anti-inflammatory role on pain processing (Averitt et al., 2019; Delaruelle et al., 2018; Nakagawa et al., 1981), estrogen's (E2) role remains controversial with reports of both pronociceptive and antinociceptive effects. The pronociceptive role of E2 has been well documented in both animal and human models. In rats, a high concentration of E2 results in upregulation of TRPV1 and nerve growth factor (NGF) expression in the central and peripheral nervous system (Wu et al., 2015; Yamagata et al., 2016), enhances allodynia in the temporomandibular joint (Wu et al., 2015), and increases formalin-induced orofacial pain behavior (Fejes-Szabo et al., 2018). E2 also has pronociceptive effects *in vitro* on pain signaling. Specifically, E2 causes a rapid Ca⁺² influx into the epithelial cells via TRPV6 channel (Irnaten et al., 2008), enhances bradykinin signaling in rat sensory neurons (Rowan et al., 2010), and regulates release of CGRP (Pota et al., 2017). On the contrary, high E2 has also been associated with analgesic effects in women with chronic pain (Hellstrom and Anderberg, 2003). In rats, E2 treatment has been reported to reduce nociceptive neural activity in cultured neurons and reduce nocifensive behaviors in ovariectomized (OVX) rats (Averitt et al., 2019). In any case, there is a clear association between trigeminal pain disorders and hormone status.

E2 may also modulate pain processing by influencing the levels of 5HT in the peripheral nervous system. It has been shown that externally administered E2 can maintain high levels of 5HT in the periphery in OVX rats (Benmansour et al., 2016). Also, E2 can increase the expression of tryptophan hydroxylase enzyme (TPH; rate limiting enzyme for 5HT synthesis), decrease the expression of serotonin reuptake transporter, and increases the binding and density of 5HT_{2A} receptors in the central nervous system (Akira Kugaya et al., 2003; Bethea et al., 2000; Moses-Kolko et al., 2003). Additionally, 5HT-evoked CGRP release is highest from the dental pulp of females in the last week of menses (Loyd et al., 2012b).

Together, these studies suggest a possible neuromodulatory role of E2 on 5HT-evoked pain signaling in trigeminal sensory neurons. As

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

Methods

Subjects

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

Vaginal cytology

Vaginal lavages were performed between 0900 AM and 1100 AM at 24-h intervals beginning 2 weeks (at least two consecutive cycles; 10 days) before testing to confirm that all female rats were cycling normally. Daily records were maintained on the stages of their cycle throughout experimental testing. Proestrus was identified as a predominance of nucleated epithelial cells and estrus was identified as a predominance of cornified epithelial cells. Diestrus 1 (or metestrus) was differentiated from diestrus 2 (or diestrus) by the presence of leukocytes (Becker et al., 2005; Loyd et al., 2008; McLean et al., 2012). When no significant differences were noted in behavior of diestrus 1 and diestrus 2 animals, these data were pooled and reported as such.

Ovariectomy

Female rats (n = 96) were deeply gas anesthetized (3% induction; 2.5% maintenance) by inhalation of Isothesia (isoflurane, USP, Henry Schein Animal Health, Dublin, OH) and a single incision was made across the abdomen. The abdominal muscle was opened and the ovary bundles were ligated with 4-0 silk sutures, excised, and removed, as previously described (White and Uphouse, 2004). The fascia was closed with 5-0 silk suture and the skin was closed with Vicryl sutures to prevent wicking. Rats were allowed 2 weeks for recovery and ovarian hormone dissipation.

Drugs

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

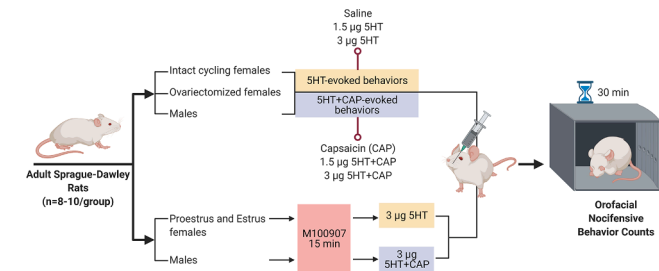
Immediately prior to use, complete Freund's adjuvant (CFA; Sigma–Aldrich) was dissolved 1:1 in 0.9% sterile saline.

Orofacial nocifensive behavior testing

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

Adult male, cycling females at each stage of the estrous cycle, and ovariectomized (OVX) females (n = 9–11 per sex, stage of estrous cycle, and experimental treatment group) were gas anesthetized and received a single intradermal injection (30-gauge needle) of either 1.5 µg/50 µL 5HT, 3 µg/50 µL 5HT, or vehicle control (0.9% sterile saline; 50 µL) into the vibrissal pad (unilateral). The number of forelimb swipes over the injected area were counted as described above as 5HT-evoked nocifensive behaviors (Timeline 1). A separate group of rats received a single intradermal injection of each dose of 5HT + a low dose of capsaicin (1.5 µg 5HT + 1 µg CAP/50 µL; 3 µg 5HT + CAP/50 µL) or control (1 µg CAP/50 µL) into the vibrissal pad (unilateral). The number of forelimb swipes over the injected area were counted as described above as the effects of 5HT on capsaicin-evoked nocifensive behaviors (Timeline 1). The dose of capsaicin was chosen based on a previous study characterizing the concentration–response of injection of capsaicin into the rat vibrissal pad and a low dose was chosen to allow for observing a potential sensitizing effect of 5HT on capsaicin (Pelissier et al., 2002).

A separate group of rats consisting of females in either proestrus or estrus and males (n = 8 per sex and experimental treatment group) received an intradermal injection of the selective 5HT_{2A} antagonist, M100907 (2 nM; 0.07 ng/100 µL) or vehicle control (100 µL 6% DMSO in 0.9% sterile saline) into the vibrissal pad (unilateral). Fifteen-minutes after the pre-treatment, female rats received an injection of 5HT (3 µg/50 µL) and the male rats received an injection of 5HT + CAP (3 µg 5HT + CAP/50 µL) at the same site (Timeline 1). Orofacial nocifensive behavior was recorded and counted as described above. The time course and concentration of M100907 was chosen for selectivity based on previous studies reporting binding affinity (K_i = 1.92 nM) of M100907 for the 5HT_{2A} receptor (<http://pdsp.med.unc.edu>).



Timeline 1. Illustration of time course of groups and treatments for behavior studies. Created with BioRender.com.

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

A separate group of male rats, OVX female rats, and cycling female rats (n = 6–7 per group) received a left vibrissal pad injection of 50 µL CFA (1:1 in sterile saline) and right vibrissal pad injection of vehicle control (50 µL 0.9% sterile saline). Twenty-four hours later, rats were briefly gas anesthetized and rapidly decapitated. Inflamed rat vibrissal pad samples were collected with four 6-mm biopsy punches (Milltex, Inc., York, PA). Tissue biopsies were placed on a filter of the cell-strainer tube (5 mL polystyrene round-bottomed tube with cell-strainer cap; BD Falcon, Franklin Lakes, NJ) and samples were centrifuged at 275 g for 20 min at 4 °C to recover the interstitial fluid. Then 1% ascorbic acid was then added to the collected fluid (to prevent 5HT degradation) and samples were stored at –20 °C. Interstitial fluid samples were then assayed in duplicate by a rat-specific Serotonin (Research) ELISA kit (IB89540; IBL Laboratories; Minneapolis, MN) and duplicate readings were averaged for analysis.

Primary culture of trigeminal ganglia neurons

Trigeminal ganglia (TG; n = 3–4 rats per 24-well plate run in triplicate) were extracted from adult OVX female rats (~200 g) immediately following decapitation. Primary neuron cultures were prepared using previously described methods (Loyd et al., 2011; Patwardhan et al., 2005). Briefly, TGs were suspended in HBSS on ice and gently washed three times. After dissociation with collagenase (5%, Worthington Biochemical Corp, Lakewood, NJ) and trypsin (1%, Sigma–Aldrich) at 37 °C, the cells were suspended in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Waltham, MA) containing 10% fetal bovine serum, glutamine, penicillin–streptomycin, nerve growth factor (NGF, 100 ng/ml; Harlan, Indianapolis, IN), and treated with mitotic inhibitors (5-fluoro-2'-deoxyuridine and uridine). Cells were then lightly dissociated using a 20-gauge followed by a 23-gauge needle and then applied to 24-well poly-D-lysine-coated plates (Corning Inc., Corning, NY) and maintained in an incubator at 37 °C and 5% CO₂.

CGRP release assay

TG primary cultures were maintained for 5 days prior to running the CGRP release assay. The assay was performed using a protocol previously described (Loyd et al., 2011). Briefly, cultures were washed with 300 µL HBSS twice to obtain baseline CGRP release. Cultures were then pretreated row-wise with either 5HT (100 µM), E2 (50 nM), M100907 (10 nM and 30 nM) a combination of 5HT + E2, or HBSS followed by stimulation with CAP (50 nM). Each treatment lasted for 15 min. The concentration of each drug was chosen based on reported known binding affinities (<http://pdsp.med.unc.edu>). Superfusate was collected following each treatment and quantitated for CGRP levels by rat-specific CGRP ELISA (Cayman Chemical, Ann Arbor, MI). All experiments were conducted in duplicate with n = 6 wells per treatment group for a total of approximately 12 wells per group.

Data analysis

All data were analyzed with GraphPad Prism software version 8.3.0 (GraphPad, San Diego, CA) or IBM SPSS Statistics version 25 (Armonk, NY). All data graphs were made with GraphPad Prism. Orofacial nocifensive behavior data were expressed as mean ± standard error of the mean (SEM) number of forelimb swipes over a 30 min time period (6 min bouts). Behavior data was analyzed by repeated measures two-way and three-way analysis of variance (ANOVA) with time as the repeated factor and both stage (males, OVX, diestrus 1 and 2 combined, proestrus, and estrus) and treatment (5HT vs saline; 5HT + CAP vs CAP; M100907 vs Vehicle) as independent factors. 5HT content in interstitial fluid was reported as mean ± SEM ng/mL of 5HT across the stages of the estrous

cycle and analyzed by ordinary two-way ANOVA. CGRP release was reported as mean \pm SEM percent baseline levels and were analyzed by paired, unpaired *t*-test, or two-way ANOVA. When unequal variance was detected, a Welch correction was applied. The Grubb's test (GraphPad Quick Calcs Online, the extreme studentized deviate method; [(mean-value)/standard deviation]) was used to exclude a single outlier within an experimental group if present. Further, animals were removed from the study when environmental factors disrupted behavior testing (e.g. significant noise in the facility was noted by the experimenter during testing). Bonferroni's correction was used to calculate *a priori* pairwise comparisons.

Results

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

We first tested whether peripheral injection of 5HT into the rat vibrissal pad evoked significant orofacial nocifensive behaviors across the estrous cycle in female rats compared to males and ovariectomized females. There was a significant three-way interaction of time by treatment by stage [$F(29.448, 116) = 1.612$; $p \leq 0.05$] and a significant main effect of time [$F(3.681, 116) = 14.558$; $p \leq 0.05$] and stage [$F(4, 116) = 2.816$; $p \leq 0.05$]. Across all animals tested (males and females)

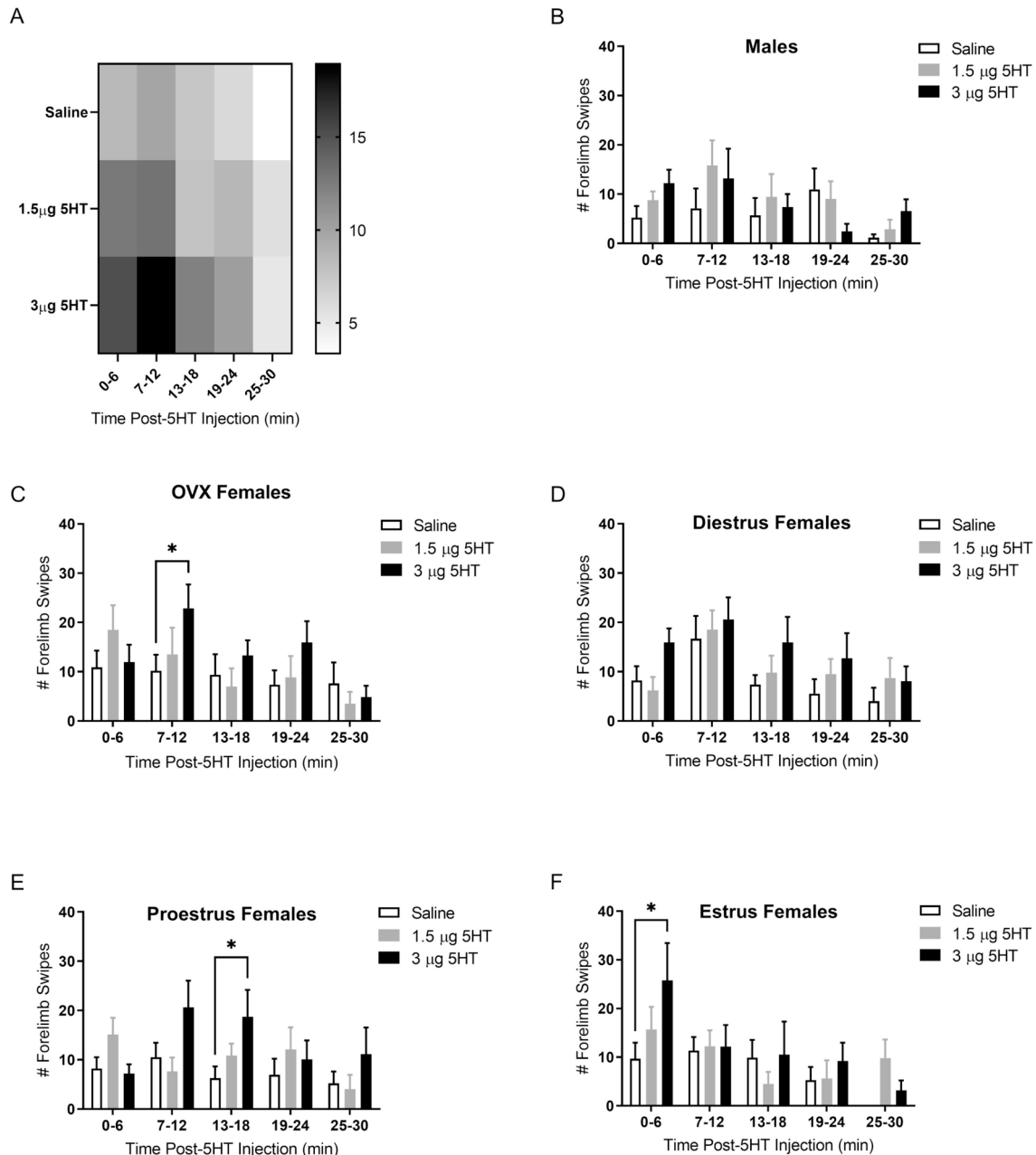


Fig. 1. Peripheral 5HT evoked significant orofacial nocifensive behaviors in female rats during proestrus and estrus only. 5HT evoked pain behaviors peaked in the initial 15 min in both male and female rats (data combined) as observed by an increase in the heatmap grayscale gradient (A). In males (B), pain behaviors evoked by 1.5 µg 5HT (grey bars) and 3 µg 5HT (closed bars) were not significantly different compared to saline vehicle control (open bars). In ovariectomized (OVX) females (C), only 3 µg 5HT evoked significant nocifensive behaviors as observed by an increase in the number of forelimb swipes. In diestrus females (D), neither dose of 5HT evoked significant pain behaviors. 3 µg 5HT evoked significant nocifensive behaviors in proestrus (E) and estrus (F) females. *Denotes a significant effect of 3 µg 5HT compared to saline at the respective time point with significance in pairwise comparisons tested at $p \leq 0.05$.

combined), nocifensive behavior characteristically peaks in the initial 0–18 min post-5HT injection in a dose-dependent manner and wanes by 30 min post-5HT injection (Fig. 1A). In male rats and diestrus females, no significant 5HT-evoked nocifensive behaviors were observed and forelimb swipes were comparable to saline injected controls (Fig. 1B–D) [$p > 0.05$]. 3 μg 5HT evoked significant nocifensive behaviors at 13–18 min in proestrus females (Fig. 1E) [$p \leq 0.05$] and at 7–12 min in ovariectomized females [$p \leq 0.05$], whereas estrus females only displayed significant pain behaviors during the initial 6 min post-5HT injection (Fig. 1F) [$p \leq 0.01$]. We did not observe any hindlimb swipes indicative of itch at the 5HT concentrations used in this study.

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

We then tested whether the nociceptive behavior can be enhanced in

the presence of 5HT by injecting 5HT with a low dose of capsaicin (CAP) and recording the behavior over a 30 min time period. There was a significant three-way interaction of time by treatment by stage [$F(29.186, 108) = 1.717$; $p \leq 0.05$] and significant two-way interactions of time by treatment [$F(7.297, 108) = 2.483$; $p \leq 0.05$], time by stage [$F(14.593, 108) = 1.991$; $p \leq 0.05$], and treatment by stage [$F(8, 108) = 2.694$; $p \leq 0.05$]. Collectively (males and females combined), nocifensive behaviors were highest in the initial 0–18 min in the 3 μg 5HT + CAP group as compared to 1.5 μg 5HT + CAP or CAP (Fig. 2A). 3 μg 5HT + CAP evoked significant nocifensive behaviors in males at the 7–12 min as compared to the CAP group (Fig. 2B) [$p \leq 0.001$]. No significant differences were observed between any 5HT + CAP treatment group and CAP in OVX females and diestrus females (Fig. 2C, 2D) [$p > 0.05$]. In proestrus females (Fig. 2E), the lower dose of 1.5 μg 5HT + CAP evoked significant nocifensive behaviors at 7–12 min [$p \leq 0.05$], whereas, in estrus females 3 μg 5HT + CAP evoked significant nocifensive behaviors

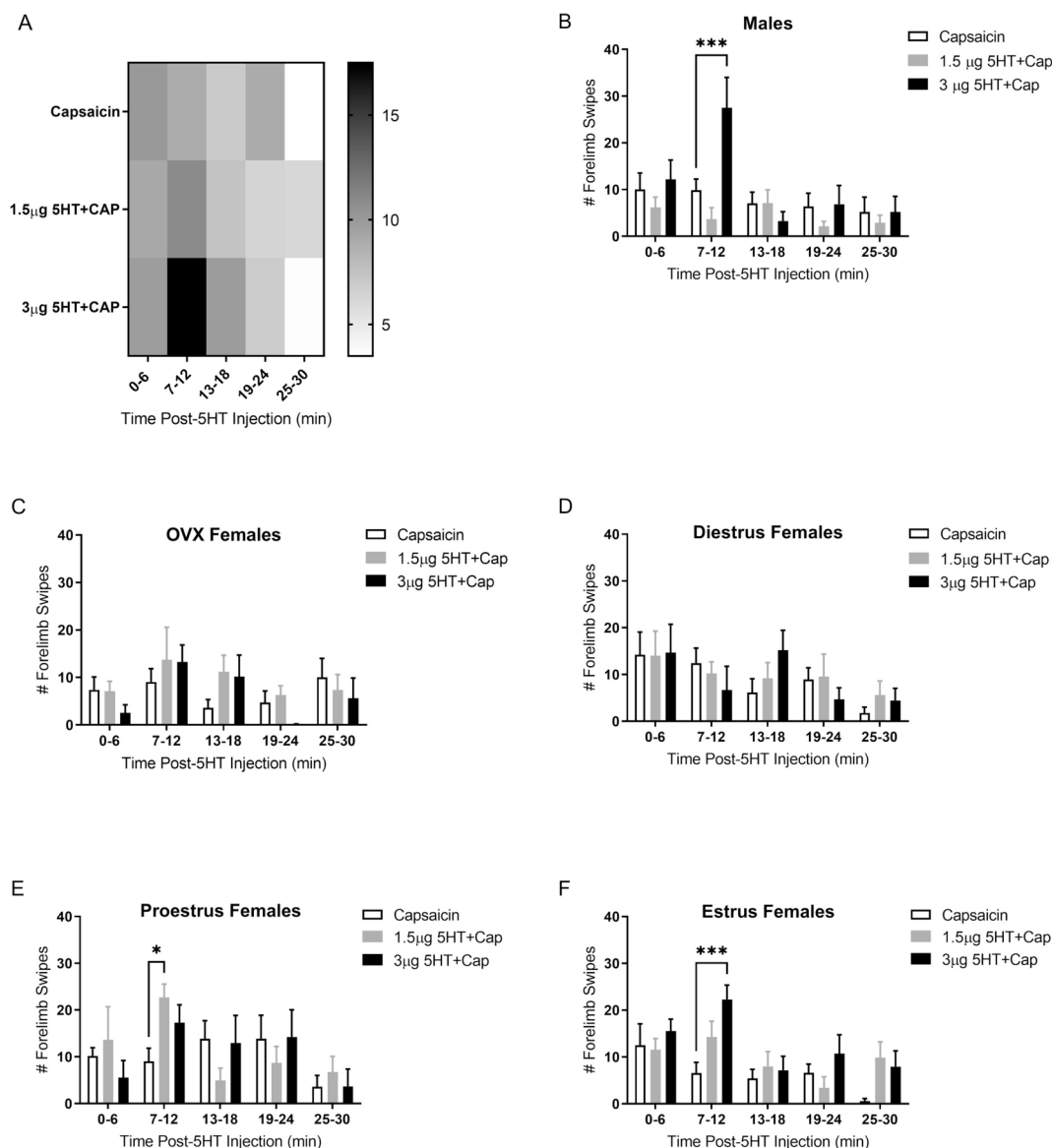


Fig. 2. Peripheral 5HT enhanced capsaicin-evoked orofacial nocifensive behaviors. 5HT-evoked pain behaviors peaked in the initial 15 min in both male and female rats (data combined) as observed by an increase in the heatmap grayscale gradient (A). 3 μg 5HT (closed bars) and not 1.5 μg 5HT (grey bars) evoked significant nocifensive behaviors in male rats (B) compared to capsaicin control (open bars) as observed by an increase in number of forelimb swipes. Neither doses of 5HT evoked significant pain behaviors in OVX females (C) and diestrus females (D). 1.5 μg 5HT evoked significant nocifensive behaviors in proestrus females (E) and 3 μg 5HT evoked significant nocifensive behaviors in estrus females (F). *Denotes a significant effect of 1.5 μg 5HT + capsaicin compared to capsaicin at the respective time point with significance in pairwise comparisons tested at $p \leq 0.05$. ***Denotes a significant effect of 3 μg 5HT + capsaicin compared to capsaicin at the respective time point with significance in pairwise comparisons reported at $p \leq 0.001$.

at 7–12 min (Fig. 2F) post-injection as compared to CAP [$p \leq 0.01$]. When data points were collapsed and only CAP-evoked behaviors were compared across males, OVX, and cycling females, there was no significant difference between any groups ($p > 0.05$).

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

Since the 5HT_{2A} receptor, an excitatory G_q protein-coupled subtype, is expressed in the trigeminal ganglia of male rats and is involved in potentiation of TRPV1 activity (Kaur et al., 2018; Loyd et al., 2011), we next determined whether the 5HT_{2A} receptor was involved in 5HT-evoked orofacial nocifensive behaviors in female rats, as indicated in males rats and the female rat hindpaw. As there was no significant effect of 5HT in males, we selected the proestrus and estrus females to receive the 5HT_{2A} antagonist in aims of reductionism. Further, because males only display 5HT-evoked nocifensive behaviors when capsaicin is present, capsaicin was added to the 5HT when treating the males with the 5HT_{2A} antagonist. Local pretreatment with the selective 5HT_{2A} receptor antagonist M100907 15 min prior to 3 μ g 5HT injection significantly reduced the number of forelimb swipes in female rats during proestrus and estrus at 13–18 min (Fig. 3A) [$p \leq 0.05$]. In male rats however,

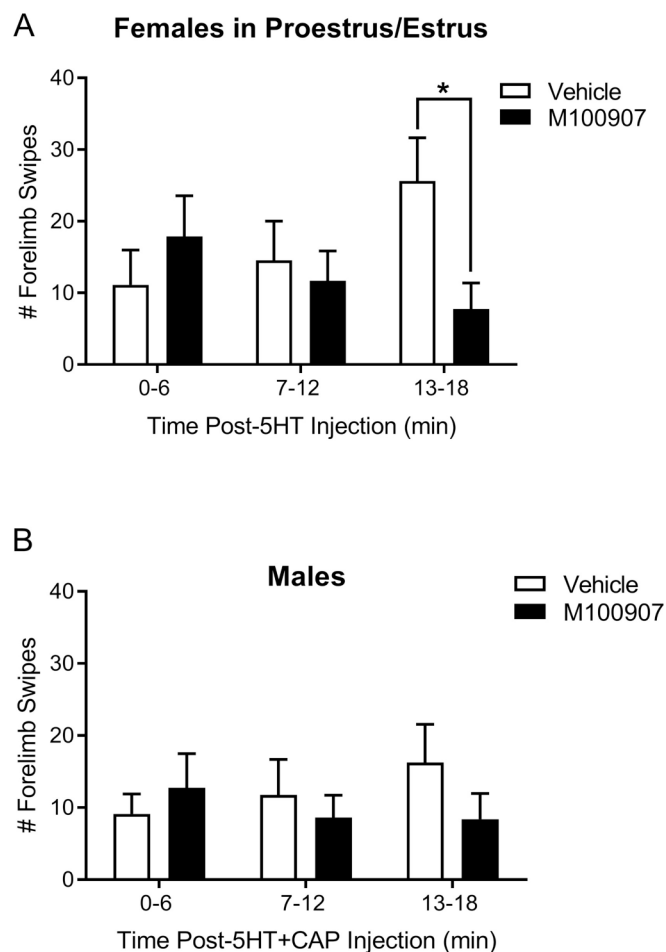


Fig. 3. Blocking the excitatory 5HT_{2A} receptor attenuated 5HT-evoked orofacial nocifensive behaviors in female rats. M100907 (closed bars) pretreatment significantly reduced 5HT-evoked nocifensive behaviors in females during proestrus or estrus compared to the vehicle (open bars) pretreated group (A). M100907 pretreatment did not attenuate 5HT + capsaicin evoked nocifensive behaviors in male rats (B). *Denotes a significant effect of M100907 compared to vehicle at the respective time point with significance in pairwise comparisons tested at $p \leq 0.05$.

pretreatment with M100907 followed by a 3 μ g 5HT + CAP injection did not significantly attenuate orofacial nocifensive behaviors (Fig. 3B) [$p > 0.05$]. The effect of 5HT_{2A} antagonism in females with capsaicin was not tested, as 5HT acts via 5HT GPCRs to sensitize TRPV1 and not via direct actions at TRPV1 (Salzer et al., 2019).

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

Next, we tested if local 5HT content in the vibrissal pad interstitial fluid following inflammation differed across males, ovariectomized females, and females in different phases of the estrous cycle by injecting CFA into the left vibrissal pad and saline in the right vibrissal pad. Twenty-four hours post-injection, cycling females in diestrus, proestrus, and estrus had significantly higher 5HT content in the interstitial fluid collected from saline treated vibrissal pad as compared to males [$p \leq 0.05$ for diestrus and $p \leq 0.01$ for proestrus/estrus]. Since the 5HT content was comparable between proestrus and estrus females, the data from these two groups was collapsed in the graph. 5HT content post-CFA evoked inflammation was comparable across all groups (Fig. 4) [$p > 0.05$].

E2 pretreatment significantly increases serotonergic potentiation of capsaicin-evoked CGRP release from trigeminal sensory neurons

Since excitatory 5HT receptor subtypes co-express with TRPV1 on male trigeminal sensory neurons leading to an enhanced CAP-evoked CGRP release (Loyd et al., 2011), we next determined if pretreatment with 5HT and E2 would further enhance this CGRP release in female trigeminal sensory neurons. Thus, we quantified the CGRP release from primary cultures of trigeminal sensory neurons pretreated with either E2 (50 nM), 5HT (100 μ M), or a combination of E2 + 5HT before being stimulated with a low concentration of CAP (50 nM). When trigeminal ganglia neurons were pretreated with only 5HT or E2 (Fig. 5A), CGRP release was comparable to the vehicle group [$p > 0.05$]. CAP evoked significant CGRP release indicative of TRPV1 activity (Fig. 5B) $F(1, 53) = [p \leq 0.05]$. There was a slight decrease in capsaicin-evoked CGRP release following 5HT pretreatment which was not significant [$p >$

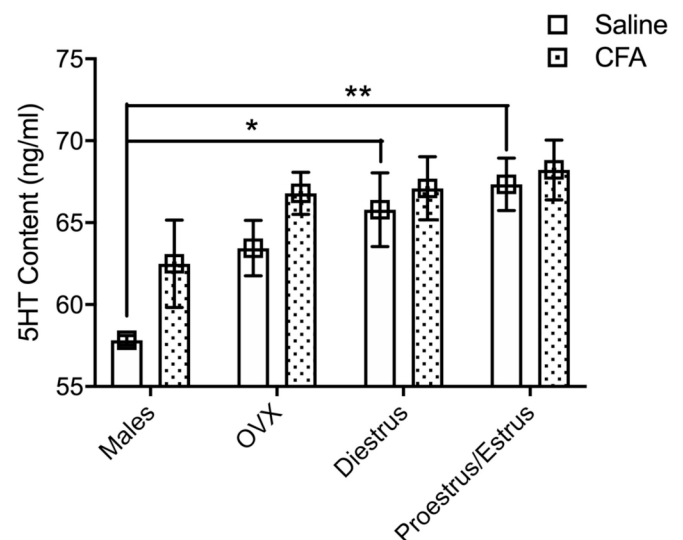


Fig. 4. Basal levels of 5HT were elevated in the interstitial fluid from cycling females. Twenty-four hours after saline (open bars) injection, 5HT content was elevated in diestrus and proestrus/estrus females as compared to males. 5HT content was not significantly different in the CFA (dotted bars) injected groups. *Denotes a significant difference between 5HT content in pairwise comparisons tested at $p \leq 0.05$. **Denotes a significant difference between 5HT content in pairwise comparisons reported at $p \leq 0.01$.

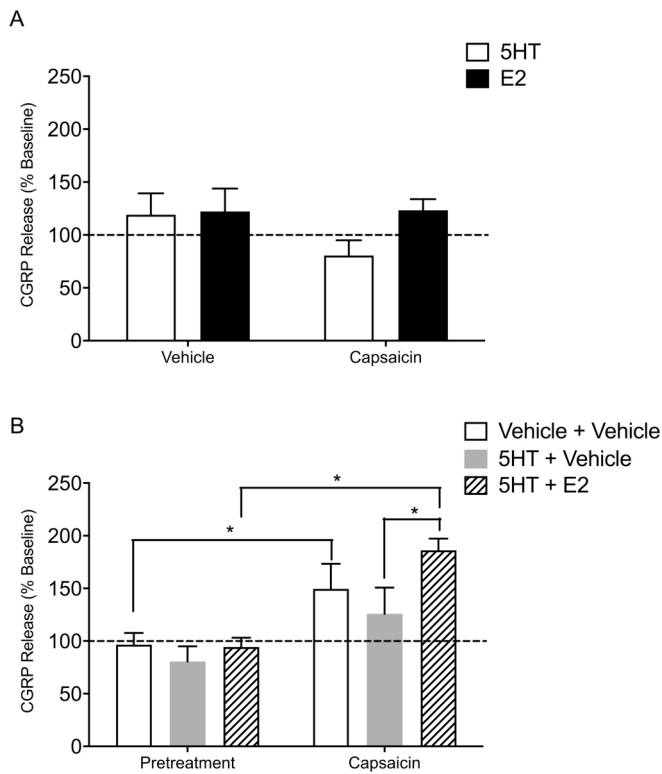


Fig. 5. E2 pretreatment significantly increased serotonergic potentiation of capsaicin-evoked CGRP release. CGRP release from female rat primary trigeminal ganglia neuron cultures following 5HT (open bars) or E2 alone (closed bars) was comparable to vehicle (A) as observed by percent change in baseline. Capsaicin evoked significant CGRP release (open bars) as compared to vehicle pretreatment. E2 + 5HT (diagonal bars) pretreatment significantly enhanced capsaicin-evoked CGRP release as compared to vehicle pretreatment and the 5HT pretreatment (grey bars) on capsaicin-evoked CGRP release (B). *Denotes a significant increase in CGRP release compared to vehicle with significance in pairwise comparisons tested at $p \leq 0.05$.

0.05]. Importantly, a combination of 5HT + E2 pretreatment followed by CAP evoked significantly higher CGRP release as compared to the 5HT + vehicle and compared to pretreatment prior to CAP stimulation (Fig. 5B) [$p \leq 0.05$]. Specifically, E2 + 5HT nearly doubled (E2 + 5HT + CAP: 186.2% whereas E2 + CAP: 123.4%; 5HT + CAP: 80.6%) the capsaicin-evoked CGRP release. When trigeminal ganglia neurons were pretreated with 10 nM or 30 nM M100907, the selective 5HT_{2A} antagonist, CGRP release was not attenuated and was comparable to the 5HT

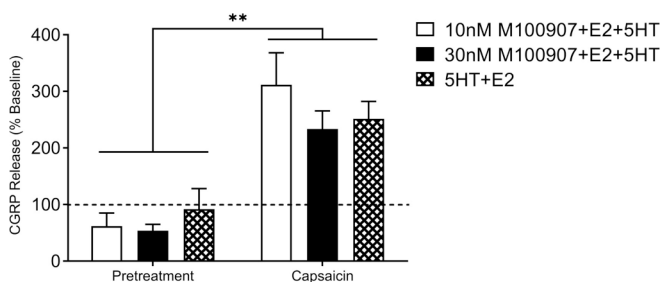


Fig. 6. Antagonism of the 5HT_{2A} receptor did not attenuate CGRP release. Pretreatment of female rat primary trigeminal ganglia neuron cultures with 10 nM (open bars) or 30 nM (closed bars) M100907 prior to treatment with E2 and 5HT did not attenuate capsaicin-evoked CGRP release compared to pretreatment (hatched bar). **Denotes significance compared to pretreatment with significance in pairwise comparisons tested at $p \leq 0.01$. ***Denotes significance compared to pretreatment in pairwise comparisons reported at $p \leq 0.0001$.

+ E2 pretreated group (Fig. 6). We were limited to M100907 concentrations < 30 nM to avoid possible activity at other receptors (<http://pdsp.med.unc.edu>).

Discussion

The role of gonadal hormones and inflammatory mediators is implicated in numerous peripheral pain conditions. Our lab has previously reported that during phases of the estrous cycle when hormones greatly fluctuate (proestrus and estrus), intraplantar 5HT injection into the hindpaw triggers significant thermal hyperalgesia and mechanical allodynia in female rats (Kaur et al., 2018). This concurs with a previous study in human tissues reporting that 5HT significantly enhances capsaicin-evoked CGRP release from human dental pulp extracted from females during the luteal phase of the menstrual cycle (Lloyd et al., 2012b). E2 replacement therapy increases plasma 5HT levels (Blum et al., 1996), binding potential of 5HT receptors (Moses-Kolko et al., 2003), and both estrogen and 5HT have been linked to multiple trigeminal pain disorders (Paredes et al., 2019). However, the role of estrogen in trigeminal pain processing is controversial, with some studies reporting a pronociceptive role while others report an antinociceptive role (Averitt et al., 2019). In the present study, we report that (1) 5HT-evoked nociceptive pain behaviors are sexually dimorphic and dependent on hormone status, (2) 5HT potentiates capsaicin-evoked pain behaviors in males and in females during proestrus and estrus, (3) cycling females have significantly higher basal peripheral 5HT levels as compared to males, (4) estrogen enhances the serotonergic neuromodulation of capsaicin-evoked CGRP release in cultured trigeminal sensory neurons, and (5) blocking the 5HT_{2A} receptor attenuates nociceptive behavior in female rats but does not affect capsaicin-evoked CGRP release in cultured trigeminal sensory neurons.

Studying pain behaviors in intact cycling females and males is essential to understanding whether gonadal hormones play a role in pain processing (Becker et al., 2005). Here, we tested the ability of two different concentrations of serotonin to evoke spontaneous nociceptive pain behaviors in intact animals across both sexes and in ovariectomized females. Similar to previous reports of the transient, nociceptive effects of peripheral 5HT (Lloyd et al., 2013), nociceptive behaviors in all tested animals (males and females combined) peaked within about 10–15 min post-5HT injection and returned to near baseline after 30 min. Interestingly, injection of 5HT into the hindpaw evokes significant pain in males (Kaur et al., 2018; Lloyd et al., 2011), while similar physiological levels of 5HT injected at the vibrissal pad did not evoke pain behaviors in males until capsaicin was also present. This indicates that trigeminal sensory neurons may be more sensitive to the sexually dimorphic effects of 5HT, supporting the numerous and diverse craniofacial pain disorders that are often 3–4 times more common in women. 5HT is also major pruritogen (Akiyama and Carstens, 2013; Jinks and Carstens, 2002) and in the present study we did not observe itch behaviors at the low concentrations of 5HT used. It is likely that increasing 5HT to 5–10 μ g (Akiyama et al., 2010; Morita et al., 2015) would induce itch behaviors and it would be interesting to observe whether itch behaviors are also modulated by estrogen.

The greatest 5HT-evoked pain behaviors were observed during proestrus and estrus. It is also notable that significant 5HT-evoked pain behaviors were observed in ovariectomized female rats. In females, dynamic estrogen fluctuations during the estrous or menstrual cycle are associated with pain modulation (Kramer and Bellinger, 2009; Mogil, 2012) and our data contribute that fluctuating gonadal hormone levels (such as during proestrus to estrus) or a loss of gonadal hormones (such as with ovariectomy) contribute to heightened sensitivity to a pronociceptive mediator. Others have also reported increased pain behaviors during these phases of the estrous cycle. Notably, treatment with prolactin (Diogenes et al., 2006) and formalin (Ibironke and Aji, 2011) display greater pain behaviors in proestrus and estrus females. Nociceptive behaviors were comparable to the saline injected control group

in males and diestrus females. For males, testosterone has been widely documented to play a major role in antinociception and analgesia in animal and human models (Archev et al., 2019; Burris et al., 1991; Frye and Seliga, 2001). It remains possible that testosterone may be playing a protective effect on serotonergic pain processing in trigeminal sensory neurons.

Interestingly, when 5HT and capsaicin were injected together, 5HT-evoked pain behaviors were then observed in males, as well as females in proestrus and estrus. In both males (only during the 7–12 min bout) and estrus females, 3 μ g 5HT evoked significant capsaicin-evoked nociceptive behaviors, whereas, in proestrus females 1.5 μ g 5HT evoked significant nociceptive behaviors. Note that neither the low dose of 5HT or capsaicin alone could not evoke significantly higher pain behaviors during proestrus, but when injected together pain behaviors emerged and were significantly higher compared to proestrus rats receiving 3 μ g 5HT. This suggests a sensitizing role of 5HT on TRPV1, causing receptor activation at a lower threshold followed by desensitization at the higher dosage. We propose that the peaking level of E2 is contributing to sensitizing the serotonergic pain mechanism during proestrus. As 5HT, via its excitatory GPCRs, can sensitize TRPV1, it is possible that a lower amount of peripheral 5HT can sensitize trigeminal nociceptors, which are then desensitized at the higher (doubled) amount of 5HT. Whereas, once E2 levels fall, there is a loss of sensitization of this mechanism and a higher amount of 5HT is required to elicit a pain response, like in males.

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

One of the several excitatory receptors of 5HT in the periphery, the 5HT_{2A} receptor, has been implicated in several pain conditions including irritable bowel syndrome, fibromyalgia, headache and migraine, and temporomandibular joint disorder (TMD) (Sugumar et al., 2004). In humans, a specific single nucleotide variation in the 5HT_{2A} receptor, rs6313, is associated with pinprick hyperalgesia (Sachau et al., 2021) and the T/T genotype of the T102C polymorphism is associated with low pain thresholds in fibromyalgia patients (Gürsoy et al., 2001). In rodents, the 5HT_{2A} receptor is involved in thermal allodynia and mechanical hypersensitivity associated with neuropathy and treatment with a 5HT_{2A} antagonist attenuated pain behaviors (Thibault et al., 2008). Based on our previous research reporting (1) localization of 5HT₁₋₃ subtypes in the trigeminal ganglia, (2) 5HT_{2A} and 5HT₃ inhibition attenuates pain behavior and pain signaling (Loyd et al., 2011), and (3) 5HT_{2A} antagonism reduces 5HT-evoked pain behaviors in the rat hindpaw (Kaur et al., 2018), we have focused on 5HT_{2A} and 5HT₃ receptors. Here, we report significant attenuation of 5HT-evoked nociceptive behaviors when females in proestrus or estrus were pre-treated with a selective 5HT_{2A} antagonist, M100907, prior to 5HT injection. 5HT_{2A} antagonism was unable to significantly reduce pain behaviors in male rats though there was a trend towards attenuation. It is of importance to note that while 5HT + capsaicin evoked pain behaviors during

the 7–12 min bout (average of 27.6 swipes), following the 5HT_{2A} antagonist we observed that the effect was spread out between the 7–12 min bout (average of 11.75 swipes) and the 13–18 min bout (average of 16.25 swipes) indicating variability in the peak of 5HT-evoked pain behaviors in males. Thus, the 5HT_{2A} antagonist may be effectively reducing pain behaviors in males, but is not being detected as significant in our data as presented across time.

While this study was limited to examining the role of the 5HT_{2A} receptor, several other 5HT receptors may also be involved and in a sexually dimorphic manner. We recently reported that 5HT_{3A} does not play a major role in sex differences in 5HT-evoked orofacial pain behaviors (Kaur et al., 2021a). In contrast, recent studies have reported a major role of the 5HT₃ receptor in mediating serotonergic pain and itch in TG and DRG neurons (Domocos et al., 2020; Kilinc et al., 2017). 5HT₄ and 5HT₇ have also recently been reported to play a role in serotonergic pain and itch (Lopez et al., 2021; Morita et al., 2015; Ohta et al., 2006). Similar to 5HT_{2A} antagonism in our study, a 5HT₇ receptor antagonist reduced pain behaviors but did not alter CGRP release in cultured trigeminal ganglia neurons (Wang et al., 2016). We speculate, as others have (Price et al., 2005; Wang et al., 2016), that an *ex vivo* culture environment does not well capitulate the complex effects of 5HT in the periphery. 5HT₆ is also an excitatory 5HT receptor, however its expression in sensory neurons is currently controversial. While our current investigations are focused on which estrogen receptors are involved in exacerbating serotonergic pain, future studies are warranted to examine the role of 5HT₄ in sex differences in serotonergic pain.

Several studies have also looked at the activation profile of TRPV1 in the presence of estrogen and inflammatory mediators. Rowan et al. demonstrated that after a short-term exposure to E2, bradykinin signaling is enhanced in trigeminal ganglia primary cultures (Rowan et al., 2010). In addition, 30 min E2 treatment has been reported to induce CGRP release in a dose dependent manner in cultured DRG cell lines (Pota et al., 2017). Similarly, we see an enhanced capsaicin-evoked CGRP release when TG primary cultures from OVX females are pre-treated with both 5HT and E2. Individually, neither 5HT nor E2 potentiate the capsaicin-evoked CGRP release. It is important to note that in our previous work in male TG neurons, 5HT potentiated capsaicin-evoked CGRP and calcium signaling (Loyd et al., 2011). However, when we looked for this effect in human nociceptors, we observed a menstrual cycle dependent effect (Loyd et al., 2012a,b). In support, excitability of TMJ neurons is highest in presence of E2 and inflammation (Flake et al., 2005). The present culture experiment specifically extracted TG neurons from OVX animals and treated them with E2 to determine the effect on 5HT-potentiated CGRP release. Interestingly, E2 treatment was required to elicit 5HT-potentiated CGRP release in OVX females at a concentration of 5HT that potentiated CGRP release in male TG neurons. Our current studies are focused on the receptor interaction underlying this sex difference.

It is possible that the effect of E2 occurs via a non-genomic effect of a membrane-bound estrogen receptor given the rapid effects of E2 on 5HT potentiated CGRP release. However, we did not see an effect of a 15 min E2 treatment alone on CGRP release. The 5HT-potentiated CGRP release occurs after E2 has been interacting in the system for 30–45 min, given our experimental design of a 15 min pretreatment with E2, followed by a 15 min treatment with E2 + 5HT, followed by a 15 min treatment with E2 + 5HT + CAP. Thus, it is also possible that a nuclear estrogen receptor is involved via a rapid signaling mechanism (Chen et al., 2021; Marino et al., 2006). We are currently focused on identifying which nuclear and/or membrane estrogen receptors (ER α , ER β , or GPER) are involved in potentiating the effects of 5HT on trigeminal sensory neurons. Previous studies have reported that ER α , ER β , and GPER are present in trigeminal sensory neurons (Chen et al., 2021; Warfvinge et al., 2020).

Overall, we report that the proinflammatory and pronociceptive mediator 5HT at the trigeminal sensory neurons triggers orofacial pain behaviors in a sexually dimorphic and estrogen-dependent manner. This

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

CRedit authorship contribution statement

Sukhbir Kaur: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Visualization, Writing - original draft, Writing - review & editing. **Hanna McDonald:** Data curation, Formal analysis, Investigation, Writing - review & editing. **Sirima Tongkhuya:** Data curation, Formal analysis, Investigation, Writing - review & editing. **Cierra M.C. Lopez:** Data curation, Formal analysis, Investigation, Writing - review & editing. **Sushmitha Ananth:** Data curation, Investigation, Writing - review & editing. **Taylor M. Hickman:** Data curation, Formal analysis, Investigation, Writing - review & editing. **Dayna L. Averitt:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

This work was supported by the National Institute of Dental & Craniofacial Research of the National Institutes of Health (grant number R15DE025970) and Texas Woman's University. The authors have no conflict of interest to declare.

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