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Sex Differences and Estrous Cycle Effects of Peripheral Serotonin-Evoked Rodent Pain Behaviors

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

Key words: pain, serotonin, estrous cycle, $5HT_{2A}$ receptor, sex differences, edema.

INTRODUCTION

Sex differences are seen in pain conditions such as fibromyalgia, irritable bowel syndrome (IBS), migraine, and temporomandibular joint disorder (TMD) pain (LeResche et al., 2003; Berkley, 1997). These conditions are more prevalent in women as compared to men with women reporting a longer duration and higher intensity of pain (Moloney et al., 2016). Based on population studies, the female: male ratio is 2:1 for migraine and 3:1 for IBS. Additionally, 80% of the treated TMD cases are females. Medical treatment of such chronic pain conditions is estimated to be approximately $635 billion per year (IOM, 2011). Thus, understanding the underlying cause of this sexual dimorphism is vital for its effective treatment.

Consistent with the hypothesis that fluctuating gonadal hormones play an important role in these differences, half of women report menstrual-associated migraine (Granella et al., 1993), pain associated with fibromyalgia is highest during the luteal phase of the menstrual cycle when estrogen levels are high (Korszun et al., 2000), and the incidence of TMD pain increases in postmenopausal women undergoing estrogen replacement therapy (Dao and LeResche, 2000). In female rats, increased visceral hypersensitivity is seen during the proestrus and estrus stages of the estrous cycle (Moloney et al., 2016). Also, the major active estrogen, 17-β estradiol, can regulate the release of the proinflammatory mediator calcitonin gene-related peptide (CGRP) in cultured cells *in vitro* (Pota et al., 2017). Thus, gonadal hormones can act through receptors present on the peripheral nerve endings to activate downstream signaling cascades that may play a role in sensitizing the nociceptors.

Another important factor that may exacerbate the pain condition is the presence of inflammation. Inflammation

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Abbreviations: 5HT, serotonin; $5HT_{2A}$ receptor, serotonin 2A receptor subtype; $5HT_3$ receptor, serotonin 3 receptor subtype; ANOVA, analysis of variance; CGRP, calcitonin gene-related peptide; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; GPCR, G protein-coupled receptor; IBS, irritable bowel syndrome; ipl, intraplantar; M100907, serotonin 2A receptor antagonist; PWL, paw withdrawal latency; TMD, temporomandibular joint disorder; TRPV1, transient receptor potential vanilloid 1 ion channel.

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involves recruitment of immune cells, mast cells, and blood platelets to the injury site, which then trigger release of a plethora of various inflammatory mediators including CGRP, serotonin (5-hydroxytryptamine; 5HT), bradykinin and prostaglandins (Rueff and Dray, 1992; Chuang et al., 2001). 5HT is a well-known mood and appetite regulatory neurotransmitter in the central nervous system. However, it is a proinflammatory and pronociceptive mediator in the periphery. Exogenous injection of 5HT into the female human masseter muscle induces local pain and allodynia (Ernberg et al., 2006) and injection of 5HT in male rat paw also elicits inflammation and hyperalgesia (Taiwo and Levine, 1992). 5HT is also shown to contribute to visceral hypersensitivity of primary afferent neurons in IBS (Sugiuar et al., 2004).

5HT can act via the seven currently known classes of 5HT receptors, $5HT_{1\text{-}7,}$ to alter electrical conductivity or to activate numerous downstream signaling cascades. Most 5HT receptors, with the exception of $5HT_3$ which is an ionotropic receptor (Hoyer et al., 2002), are metabotropic and act via G protein-coupled receptors (GPCRs). Some of the 5HT GPCRs can trigger pain via activating signaling cascades known to sensitize the transient receptor potential vanilloid 1 (TRPV1) ion channel, a thermosensor expressed by a major subset of sensory neurons that is activated by noxious heat (> 42 °C), capsaicin, and protons. Activation of TRPV1 channels causes a transient calcium influx and subsequent release of pronociceptive mediators, including CGRP (Caterina et al., 1997; Tominaga et al., 1998), to trigger and contribute to peripheral sensitization. Of the 5HT receptors localized on nociceptors, the $5HT_{2A}$ appears to be the most involved in pain and sensitization of TRPV1 (Sugiuar et al., 2004; Okamoto et al., 2005; Sasaki et al., 2006; Loyd et al., 2012a,b, 2011). Application of topical capsaicin in humans is associated with a greater increase in pain intensity in females as compared to males (Frot et al., 2004). In support, higher intensity of pain is reported in females in the menstrual phase compared to the luteal phase after an intradermal capsaicin injection (Gazerani et al., 2005).

Serotonin increases capsaicin-evoked CGRP release from rat sensory neurons via the peripheral $5HT_{2A}$ and $5HT_3$ receptors (Loyd et al., 2011, 2012c). These receptors are co-expressed with TRPV1 on sensory neurons providing an anatomical substrate for enhancing pain signaling in these cells (Loyd et al., 2011). This potentiation of TRPV1 by 5HT has also been demonstrated in human nociceptors in dental pulp extracted specifically during the luteal phase of the menstrual cycle (Loyd et al., 2012a), indicating a potential modulatory role of hormones on this pain mechanism. Reports from a variety of fields indicate that estrogen is modulating physiology via 5HT; including vasodilation, clotting, recruitment of immune cells, gastrointestinal motility, lordosis, and initiation of uterine contractions (see Rybaczyk et al., 2005; Uphouse et al., 2011). The degree of importance of 5HT in these functions is illustrated by the observation that 99% of 5HT is found outside the central nervous system. Specifically, an increase in estrogen leads to an increase in plasma 5HT (Blum et al., 1996) and tryptophan hydroxylase (rate-limiting enzyme for 5HT synthesis) (Bethea et al., 2000), while both reducing and antagonizing the serotonin reuptake transporter (Pecins-Thompson et al., 1998; Ofir et al., 2003), in humans and macaques. As 5HT is proinflammatory and pronociceptive in the periphery, the effects of fluctuating estrogen on the serotonergic system have clear implications in the prevalence of pain disorders involving 5HT in women.

Despite the prevalence of pain disorders in women, the underlying mechanisms linking fluctuating ovarian hormone levels with pain remain elusive. The vast majority of pain conditions that are more prevalent in women share a role of 5HT in the underlying pain mechanism, including migraine (Ferrari et al., 2001; Chen and Ashcroft, 2008), TMD (Okamoto et al., 2005), IBS (Cremonini et al., 2003), and fibromyalgia (Hauser et al., 2009; Andrews and O'Neill, 2011). To date, no study has examined the relationship between the effects of peripheral 5HT and stage of menstrual/estrous cycle on pain behaviors in females. Here we hypothesized that peripheral 5HT evokes greater pain behaviors during periods of the rat estrous cycle when gonadal hormones are in flux.

EXPERIMENTAL PROCEDURES

Subjects

A total of 49 adult male and 173 adult female Sprague–Dawley rats (250–350 g; Charles River Laboratories, Wilmington, MA) were used in these experiments. Rats were separated by sex and pair-housed in a 12:12-h light: dark cycle with *ad libitum* access to food and water. All studies were approved by the Texas Woman's University Institutional Animal Care and Use Committee and conform to federal guidelines and guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain. This study was conducted in strict compliance with the Animal Welfare Act, implementing Animal Welfare Regulations, and the principles of the Guide for the Care and Use of Laboratory Animals.

Vaginal cytology

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

Ovariectomy

Female rats (n = 25) were deeply gas anesthetized (3% induction; 2.5% maintenance) by inhalation of Isothesia