Published in final edited form as:

Neuroscience. 2012 February 17; 203: 207–215. doi:10.1016/j.neuroscience.2011.12.019.

Antihyperalgesic Effects of Anti-Serotonergic Compounds on Serotonin- and Capsaicin-evoked Thermal Hyperalgesia in the Rat

Dayna R. Loyd¹, Paul B. Chen¹, and Kenneth M. Hargreaves^{1,2}

¹Department of Endodontics University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas 78229-3900

²Department of Pharmacology University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas 78229-3900

Abstract

The peripheral serotonergic system has been implicated in the modulation of an array of pain states, from migraine to fibromyalgia, however the mechanism by which serotonin (5HT) induces pain is unclear. Peripherally released 5HT induces thermal hyperalgesia, possibly via modulation of the transient receptor potential V1 (TRPV1) channel, which is gated by various noxious stimuli, including capsaicin. We previously reported *in vitro* that 5HT increases calcium accumulation in the capsaicin-sensitive population of sensory neurons with a corresponding increase in proinflammatory neuropeptide release, and both are antagonized by pretreatment with 5HT_{2A} and 5HT₃ antagonists, as well as the anti-migraine drug sumatriptan. In the current study, we extended these findings *in vivo* using the rat hindpaw thermal assay to test the hypothesis that peripheral 5HT enhances TRPV1-evoked thermal hyperalgesia that can be attenuated with 5HT_{2A} and 5HT₃ receptor antagonists, as well as sumatriptan. Thermal hyperalgesia and edema were established by 5HT injection (0.1-10 nmol/100 μ L) into the rat hindpaw and the latency to paw withdrawal (PWL) from noxious heat was determined. Rats were then pretreated with either 5HT prior to capsaicin (3 nmol/10 μ L), the 5HT_{2A} receptor antagonist ketanserin or the 5HT₃ receptor antagonist granisetron (0.0001-0.1 nmol/100 μ L) prior to 5HT and/or capsaicin, or the 5HT_{1B/1D} receptor agonist sumatriptan (0.01-1 nmol/100 μ L) prior to capsaicin and PWL was determined. We report that 5HT pretreatment enhances TRPV1-evoked thermal hyperalgesia, which is attenuated with local pretreatment with ketanserin, granisetron or sumatriptan. We also report that peripheral 5HT induced a similar magnitude of thermal hyperalgesia in male and female rats. Overall, our results provide *in vivo* evidence supporting an enhancing role of 5HT on TRPV1-evoked thermal hyperalgesia, which can be attenuated by peripheral serotonergic intervention.

Keywords

hyperalgesia; 5HT; 5HT receptors; granisetron; ketanserin; sumatriptan

Correspondence to: Dayna R. Loyd, MSCI, Ph.D. Battlefield Pain Management Research Area US Army Institute of Surgical Research 3698 Chambers Pass Fort Sam Houston, Texas 78234 dayna.r.loyd@us.army.mil Phone: 210-539-9338 Fax: 210-539-1460.

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The authors report no conflicts of interest.

It is estimated that up to 56 million American adults, or about 28% of the adult population, experience some form of persistent pain (Brennan et al., 2007). Persistent pain, such as migraine and fibromyalgia, is often poorly managed and is an expensive burden for both taxpayers and the healthcare system. It is becoming increasingly clear that the neurotransmitter serotonin (5HT) plays a modulatory role in various acute and persistent pain states (Hargreaves and Shephard, 1999, Ernberg et al., 2000b, Herken et al., 2001, Parada et al., 2001, Sommer, 2004, Ernberg et al., 2006). Therapeutics targeting the 5HT system are currently being examined in clinical trials for their ability to treat migraine (Ferrari et al., 2001, Chen and Ashcroft, 2008), fibromyalgia (Hauser et al., 2009), and irritable bowel syndrome (Cremonini et al., 2003). However, despite the successful development of pain therapies targeting the 5HT system, the precise peripheral mechanisms of 5HT in pain states remains unclear.

The vast majority of 5HT in the mammalian body is located in peripheral tissues where 5HT is actively taken up and released with other chemical mediators by platelets, mast cells, and immune cells (Sommer, 2004). 5HT levels are increased during inflammation occurring in rats after thermal injury (Sasaki et al., 2006), in humans with joint movement pain (Kopp, 1998) and in human muscle associated with pain and allodynia (Ernberg et al., 2000b, Ernberg et al., 2000c). Peripheral 5HT administration evokes inflammation and hyperalgesia in both humans (Babenko et al., 2000, Ernberg et al., 2000b, Ernberg et al., 2000c, Ernberg et al., 2006) and animals (Taiwo and Levine, 1992, Tokunaga et al., 1998, Okamoto et al., 2002), as well as pruritogenic effects (Jinks and Carstens, 2002, Akiyama et al., 2010) that may play a role in hyperalgesia. Inflammation in the rat hindpaw induces a dose-dependent increase in peripheral 5HT levels (Nakajima et al., 2009) and formalin- induced nociception is attenuated by local administration of 5HT receptor antagonists (Parada et al., 2001). Administration of 5HT_{2A} and 5HT₃ agonists mimic 5HT-induced hyperalgesia in rats (Tokunaga et al., 1998, Obata et al., 2000, Ohta et al., 2006), while antagonists reduce hyperalgesia (Tokunaga et al., 1998, Obata et al., 2000, Sasaki et al., 2006). On the other hand, 5HT_{1B/1D} receptor agonists significantly attenuate neurogenic inflammation (Carmichael et al., 2008) and hyperalgesia (Bingham et al., 2001, Nikai et al., 2008) presumably by inhibiting release of neuropeptides (Moskowitz and Buzzi, 1991). Together, these studies indicate that the complexity of the peripheral 5HT system in various pain states may be due, in part, to activation of a broad range of 5HT receptor subtypes. However, it is possible that these various 5HT receptor subtypes may regulate common transduction systems, leading to convergence of peripheral 5HT pain mechanisms.

The transient receptor potential V1 channel (TRPV1) plays a critical role in inflammatory pain states by mediating sensory neuron activation by chemical and thermal stimuli (Caterina et al., 1997, Caterina et al., 2000, Davis et al., 2000, Voets et al., 2004) and factors released during inflammation sensitize TRPV1 to enhance hyperalgesia (Cesare et al., 1999, Pingle et al., 2007). Importantly, there is mounting evidence of a role of 5HT in modulating TRPV1 function. 5HT reportedly increases sensory neuron membrane excitability to thermal stimuli and enhances capsaicin- and heat-evoked currents (Sugiuar et al., 2004, Ohta et al., 2006), while 5HT depletion attenuates visceral pain with a corresponding reduction in TRPV1 activation (Qin et al., 2010). 5HT enhances intracellular calcium accumulation in capsaicin-sensitive sensory neurons (Ohta et al., 2006, Loyd et al., 2011) and enhances capsaicin-evoked calcitonin gene-related peptide (CGRP) release, while 5HT_{2A} and 5HT₃ antagonists, as well as the anti-migraine drug sumatriptan, attenuate 5HT enhancement of capsaicin-evoked CGRP release (Loyd et al., 2011).

Despite the growing evidence of a modulatory role of 5HT on TRPV1 function, it remains unclear whether 5HT enhances TRPV1-evoked thermal hyperalgesia *in vivo* or whether targeting peripheral 5HT receptors can reduce capsaicin-evoked thermal hyperalgesia. We

hypothesized that 5HT enhances TRPV1-evoked thermal hyperalgesia that can be attenuated with 5HT_{2A} and 5HT₃ receptor antagonists, as well as the 5HT_{1B/1D} agonist sumatriptan. Using behavioral assays in the rat hindpaw, we (1) established 5HT evoked thermal hyperalgesia and edema, (2) evaluated whether 5HT pretreatment enhanced TRPV1-evoked thermal hyperalgesia and (3) determined whether the 5HT_{2A} receptor antagonist ketanserin, the 5HT₃ receptor antagonist granisetron, or the 5HT_{1B/1D} receptor agonist sumatriptan treatment could reduce 5HT- and TRPV1-evoked thermal hyperalgesia.

EXPERIMENTAL PROCEDURES

Subjects

A total of 118 adult (250-350 g) intact male, 34 intact female and 32 ovariectomized (OVX) female Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were used in these experiments. Rats were housed for at least 5 days prior to study with *ad libitum* access to food and water. These studies were performed in compliance with the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio and conform to federal guidelines and guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain.

Drugs

Drugs (Sigma-Aldrich, St. Louis, MO) were dissolved and stored at 4°C in stock concentration form and diluted in buffered saline (experimental vehicle) immediately prior to use, except serotonin hydrochloride which was both dissolved and diluted immediately prior to each use. Capsaicin (TRPV1 agonist) was dissolved in ethanol stock, with final dilutions containing <0.5% ethanol. Serotonin hydrochloride, sumatriptan succinate (5HT_{1B/1D} receptor agonist) and granisetron hydrochloride (5HT₃ receptor antagonist) were dissolved in double-distilled water. Ketanserin (+)-tartrate salt (5HT_{2A} antagonist) was dissolved in dimethyl sulfoxide (DMSO). Ritanerlin (5HT_{2A} antagonist) was dissolved in saline containing 5% DMSO and 5% Tween.

Behavioral Testing

Paw withdrawal latencies to a noxious thermal stimulus were determined using the Paw Thermal Stimulator (Univ. California San Diego). Rats were placed in a clear Plexiglas box resting on an elevated glass plate maintained at 30°C. Following acclimation, a radiant beam of light was positioned under the hindpaw and the average time over three trials for the rat to remove the paw from the thermal stimulus was electronically recorded in seconds as the paw withdrawal latency (PWL). The intensity of the beam was set to produce basal PWL's of approximately 10-12 seconds. A maximal PWL of 20 seconds was used to prevent excessive tissue damage due to repeated application of the thermal stimulus. All studies were conducted by an observer blind to the experimental condition.

Serotonin-evoked Thermal Hyperalgesia—To determine 5HT-induced thermal hyperalgesia in male and female rats, 5HT (0.01 – 10 nmol/100 µL; n=6-10 each) or saline vehicle was administered by intraplantar (ipl) injection using a 30 gauge syringe into the hindpaw immediately following collection of basal PWL measures. PWL levels were then reassessed at 15 min and 30 min following injections based on previous studies (Sufka et al., 1992, Taiwo and Levine, 1992, Tokunaga et al., 1998). An additional group of male rats received pretreatment with either ketanserin (0.0001-0.1 nmol/100 µL), granisetron (0.0001-0.1 nmol/100 µL) or saline vehicle 15 min prior to 5HT (10 nmol/100 µL) injections (n=6-8/group). In a separate group, 0.1 nmol/100 µL ketanserin or 0.1 nmol/100 µL granisetron (n=6 each) were injected into the contralateral hindpaw prior to 5HT injections to confirm a peripheral action of these drugs. In addition, a preliminary group of

12 male rats were used to determine the optimal methodology for administration of 5HT injections followed by behavioral testing. Six rats received brief inhalation anesthesia (Isoflurane; Butler Animal Health Supply, Dublin, OH) and 6 rats were placed under brief restraint in an individual plastic restrainer (Braintree Scientific, Braintree, MA) during ipl injection. Based on the results, all studies were then carried out using brief restraint for injections. The opposite hindpaw of rats receiving peripherally restricted compounds were used in additional experiments within 1-2 weeks to reduce the total number of animals used, except with animals receiving isoflurane.

Capsaicin-Evoked Thermal Hyperalgesia—5HT (0.1 or 10 nmol/100 μ L; n=6 each) was injected ipl to the hindpaw 10 min prior to capsaicin (3 nmol/10 μ L). The time course was chosen as 5HT-evoked thermal hyperalgesia peaks at 10-15 minutes and the dose of capsaicin was chosen to permit analysis of potential increased reduction in PWL based on previous studies (Gilchrist et al., 1996, Patwardhan et al., 2006). In a separate group of animals, ketanserin (0.1 nmol/100 μ L), granisetron (0.1 nmol/100 μ L), or saline was injected into the same hindpaw 15 min prior to 5HT and capsaicin (n=6/group). Additional groups received ketanserin (0.1 nmol/100 μ L), granisetron (0.1 nmol/100 μ L), sumatriptan (0.1 nmol-1 nmol/100 μ L), or ritanserin (100 nmol/100 μ L) prior to capsaicin alone to detect a potential effect of these drugs on capsaicin-evoked thermal hyperalgesia (n=6/group). Ritanserin (100 nmol/100 μ L; n=5) was also given alone to determine effects on basal PWL.

Plethysmometry

Rat hindpaw edema was quantified by electronically measuring changes in paw size by volume displacement with a plethysmometer (paw volume meter; Ugo Basile, Collegeville, PA) and data were reported as a percent change from baseline. Measures of paw volume were observed every 10 min for 50 min following injection of 5HT (0.1-10 nmol/100 μ L) or saline (n=4/group). To determine if ketanserin or granisetron could attenuate edema, an additional group received either ketanserin (0.1 nmol/100 μ L), granisetron (0.1 nmol/100 μ L) or saline 15 min prior to 5HT (10 nmol/100 μ L; n=4/group).

Data Analysis

Data were analyzed by using GraphPad Prism software version 5 (GraphPad, San Diego, CA) and data are presented as paw withdrawal latency in seconds or percent change from baseline. Significant outliers were identified for exclusion with the Grubbs' test (GraphPad Quick Calcs Online, the extreme studentized deviate method; [(mean-value)/standard deviation]) used to detect outliers over 2 standard deviations from the mean. Only one outlier was removed from each group when detected; reported numbers of animals per group exclude the outlier. Experimental data were analyzed by one-way or two-way ANOVA and individual groups were compared using Bonferroni *post hoc* tests. The statistical significance was tested at $p < 0.05$. Coefficient of variation was calculated by dividing the standard deviation by the mean.

RESULTS

Precision analysis of intraplantar injection methodology

As 5HT produces a rapid, transient effect on thermal hyperalgesia requiring testing within 15 minutes following injection, it is important to employ an injection method that generates consistent test values within a few minutes of injection. Therefore, we first compared the precision of PWL values in rats that received an ipl injection of 5HT under brief inhalation anesthesia vs. under brief restraint without anesthesia. Precision was measured by calculating the coefficient of variation (standard deviation/mean), with smaller values

representing a higher degree of precision. We found that animals receiving gas anesthesia recorded higher CV values in PWL 15 and 30 minutes following 5HT injection compared to animals given injections under restraint, while basal PWL were comparable (Table 1). Therefore, all of the following experiments were conducted with injections administered under brief restraint without anesthesia to optimize precision of results and minimize number of rats used.

5HT-evoked thermal hyperalgesia and edema in male rats

We next characterized the dose at which 5HT triggered thermal hyperalgesia and edema in the rat hindpaw. 5HT at doses of 1 and 10 nmol/100 μ L, but not 0.1 nmol, induced a significant reduction in PWL, indicative of increased thermal sensitivity, 15 minutes following ipl administration [$F(3,86)=4.053$; $p<0.05$] (Figure 1A) compared to saline vehicle. Basal PWL's were comparable between all groups ($p>0.05$) and are presented as a composite baseline. Latencies returned to basal levels 30 min post-injection. A significant increase in paw volume, indicative of edema, was observed for up to 50 min following injection of 10 nmol 5HT compared to saline [$F(3,55)=30.36$; $p<0.05$] (Figure 1B). The lower (0.1 – 1 nmol) doses of 5HT did not evoke a significant change in paw volume.

5HT evokes comparable thermal hyperalgesia in intact and OVX female rats

To our knowledge, no study has evaluated a sex difference in peripheral 5HT evoked thermal hyperalgesia. To address this gap, we next characterized the dose at which 5HT evoked thermal hyperalgesia in intact and OVX female rats. The ipl injection of 5HT at doses of 1 – 10 nmol/100 μ L evoked a significant decrease in PWL in both intact female rats [$F(4,52)=5.933$; $p<0.05$] (Figure 2A) and OVX rats [$F(4,46)=9.554$; $p<0.05$] (Figure 2B), while 0.01 - 0.1 nmol/100 μ L 5HT did not alter latencies compared to vehicle controls in either groups. There was no significant difference in thermal hyperalgesia between intact males, intact females and OVX females [$F(2,23)=0.4722$; $p>0.05$], therefore only male rats were used in the next series of experiments.

Ketanserin and granisetron attenuate 5HT-evoked thermal hyperalgesia

We next examined whether pretreatment with the 5HT_{2A} antagonist ketanserin or the 5HT₃ antagonist granisetron could attenuate 5HT-evoked thermal hyperalgesia. Injection of ketanserin (0.001 - 0.1 nmol/100 μ L; ipl) 15 minutes prior to 5HT injection significantly reversed 5HT-evoked thermal hyperalgesia [$F(5,76)=10.09$; $p<0.05$] (Figure 3A), while the lower dose of ketanserin (0.0001 nmol/100 μ L) did not have an effect on PWL. Also, administration of ketanserin into the contralateral paw did not alter PWL values of the hyperalgesic paw supporting a peripheral action of ketanserin. Injection of granisetron 15 minutes prior to 5HT injection induced a dose-dependent increase in PWL (Figure 3B), with a significant attenuation of 5HT-evoked thermal hyperalgesia at the 0.01 – 0.1 nmol/100 μ L doses [$F(5,54)=3.194$; $p<0.05$]. Similar to ketanserin, there was no effect of contralateral administration of granisetron on the hyperalgesic paw. Additionally, pretreatment with ketanserin, but not granisetron, 15 minutes prior to 5HT injection, significantly attenuated 5HT-evoked increase in paw volume compared to saline vehicle [$F(2,35)=15.06$; $p<0.05$] (Figure 4).

Serotonin enhances capsaicin-induced thermal hyperalgesia which is blocked by ketanserin and granisetron

Our previous studies have reported that 5HT enhances capsaicin-evoked calcium accumulation and proinflammatory neuropeptide release in cultured sensory neurons, which is attenuated with ketanserin and granisetron pretreatment (Loyd et al., 2011). Therefore, we next examined whether 5HT enhances capsaicin-evoked thermal hyperalgesia *in vivo* and if

hyperalgesia can be attenuated with ketanserin and granisetron pretreatment. We report that 10 nmol/100 μ L, but not 0.1 nmol, 5HT pretreatment 10 minutes prior to capsaicin (3 nmol/10 μ L; ipl) enhances capsaicin-evoked thermal hyperalgesia and significantly prolongs hyperalgesia at 20 and 30 minutes following capsaicin injection compared to saline vehicle pretreatment [$F(2,54)=27.16$; $p<0.05$] (Figure 5A). Ketanserin pretreatment 15 minutes prior to 5HT and capsaicin injection blocked the ability of 5HT to sensitize and prolong capsaicin-evoked thermal hyperalgesia (Figure 5B, grey bars). A similar effect was observed with granisetron pretreatment (Figure 5B, black bars) [$F(2,60)=0.905$; $p>0.05$]. There was no significant effect of either ketanserin or granisetron on capsaicin alone [$F(2,60)=1.993$; $p>0.05$] (Figure 5C).

We also tested the 5HT_{2A} antagonist ritanserin on basal paw withdrawal latencies and capsaicin-evoked thermal hyperalgesia and found that ritanserin mimicked 5HT-evoked thermal hyperalgesia, peaking earlier at 5-10 minutes [$F(6,36)=6.823$; $p>0.05$] (Figure 6A) compared to vehicle (5% DMSO/Tween in saline). When given ipl 15 minutes prior to capsaicin, ritanserin enhanced and prolonged TRPV1-evoked thermal hyperalgesia (Figure 6B). This enhancement was greatest at 20 minutes post-injection [$F(2,78)=4.196$; $p<0.05$], in a similar, time-dependent manner as 5HT. Unlike 5HT-evoked hyperalgesia, pretreatment with the 5HT_{2A} receptor antagonist ketanserin had no significant effect on ritanserin-evoked thermal hyperalgesia [$F(1,90)=0.4026$] (data not shown).

Sumatriptan attenuates TRPV1-evoked thermal hyperalgesia

Similar to ketanserin and granisetron, we have previously reported that the anti-migraine drug sumatriptan, a 5HT_{1B/1D} agonist, also attenuates TRPV1 function in cultured sensory neurons (Loyd et al., 2011). Here we examined this finding *in vivo* by injecting sumatriptan ipl 15 minutes prior to capsaicin to detect a potential attenuation of TRPV1-evoked thermal hyperalgesia. Peripheral sumatriptan at a dose of 1 nmol/100 μ L significantly attenuated thermal hyperalgesia 10 min, but not 5 min, following capsaicin injection [$F(5,70)=17.80$; $p<0.05$] (Figure 7). The lower doses of sumatriptan had no significant effect on capsaicin-evoked thermal hyperalgesia.

DISCUSSION

In the present study, we report that: (1) 5HT-evoked thermal hyperalgesia was comparable between intact male, intact female and OVX female rats, (2) 5HT pretreatment enhanced and prolonged TRPV1-evoked thermal hyperalgesia (3) and ketanserin and granisetron treatment attenuated 5HT-enhancement of TRPV1-evoked thermal hyperalgesia, while sumatriptan attenuated TRPV1-evoked thermal hyperalgesia. We also report that latencies recorded at 15 and 30 minutes post-injection administered under inhalation anesthesia were more variable than injections given under brief restraint without anesthesia.

5HT evoked rapid and transient thermal hyperalgesia peaking between 10-15 minutes and returning to basal responses by 30 minutes, similar to previous studies (Sufka et al., 1992, Taiwo and Levine, 1992). As approximately 3.5 μ g 5HT is released locally following thermal injury (Sasaki et al., 2006) and inflammation induces a 4-fold increase in 5HT concentrations (Doak and Sawynok, 1997, Parada et al., 2001, Nakajima et al., 2009), the effects of these levels of 5HT are physiologically relevant. In support, 10 nmol/50 μ L 5HT ipl enhances formalin-induced flinching (Doak and Sawynok, 1997). We also report that 5HT induces a 140% increase in paw volume indicating significant hindpaw edema. Interestingly, while 1 nmol 5HT evoked significant thermal hyperalgesia, the edema observed at this dose was not significantly different than saline or 0.1 nmol 5HT. This may implicate differential mechanisms involved in 5HT-evoked thermal hyperalgesia and 5HT-evoked edema. Additionally, peripheral 5HT has been reported to have pruritogenic

properties (Jinks and Carstens, 2002, Akiyama et al., 2010) that may play a role in the observed sensitivity to thermal stimuli. Indeed, licking/biting and shaking of the hindpaw was typically observed during the first five minutes following 5HT injection. Future studies characterizing the interaction between 5HT-evoked nocifensive behaviors (Klein et al., 2011) and 5HT-evoked hyperalgesia are important to identifying the potential role of itch in hyperalgesia.

We did not find any sex differences in 5HT-evoked thermal hyperalgesia; neither did we find a difference in 5HT-evoked thermal hyperalgesia between intact versus OVX female rats. Interestingly, it has been reported that 5HT synthesis (Berman et al., 2006) and 5HT receptor expression (Gundlach et al., 1999, Zhang et al., 1999) is altered over the estrous cycle. It is possible that the model of hindpaw 5HT inflammation is not an optimal model to detect potential sex differences or that the central effects of 5HT are sexually dimorphic while the peripheral mechanisms are similar in both sexes. Future studies addressing this question in other models such as trigeminal pain models may be appropriate and are warranted given the high prevalence of pain disorders in women.

We previously reported that both the 5HT_{2A} and 5HT₃ receptors are involved in 5HT-evoked proinflammatory neuropeptide release *in vitro* (Loyd et al., 2011). Here we found that both ketanserin and granisetron reversed 5HT-evoked thermal hyperalgesia *in vivo*. Previous studies have reported similar attenuation of 5HT-evoked nocifensive pain (Sufka et al., 1992), formalin-evoked flinch (Doak and Sawynok, 1997) and CFA-evoked orofacial nocifensive behavior (Okamoto et al., 2005). It was previously reported that ketanserin had no significant effect on mechanical hyperalgesia (Taiwo and Levine, 1992), which may indicate that 5HT_{2A} receptors are specifically localized on nociceptors involved in thermal hyperalgesia, such as the TRPV1-expressing subset as supported by our previous study (Loyd et al., 2011). Importantly, granisetron evoked a steady, dose-response attenuation of thermal hyperalgesia, which is consistent with its ability to block 5HT-enhancement of proinflammatory neuropeptide release *in vitro* (Loyd et al., 2011), indicating that it may serve as a valuable pain therapeutic; consistent with other studies in both animal models and humans (Ernberg et al., 2000a, Sung et al., 2008).

Ketanserin attenuated 5HT-evoked edema, similar to a previous study (Sufka et al., 1992), while granisetron administration had no effect on edema, indicating that different 5HT receptor systems control different mechanisms of pain and inflammation. This is supported by reports of 5HT_{2A} mRNA expression in blood vessels (Ullmer et al., 1995) and 5HT₃ receptor expression on nociceptors (Zeitz et al., 2002). Importantly, neither drug had an effect on the PWL of the 5HT injected paw when injected in the contralateral paw, providing evidence of a peripheral site of action of these drugs on 5HT-evoked thermal hyperalgesia. Another study reported that systemic ketanserin attenuates 5HT-evoked thermal hyperalgesia, while a 5HT₃ antagonist had no effect (Tokunaga et al., 1998). Together, these studies support a peripheral role of the 5HT₃ receptor in modulating pain, while it appears that the 5HT_{2A} receptor is involved in both central and peripheral control of pain.

Our previous studies reported that 5HT enhances capsaicin-evoked calcium accumulation and proinflammatory neuropeptide release (Loyd et al., 2011), however no studies have examined the effect of 5HT on TRPV1-evoked thermal hyperalgesia. Here we found that 5HT enhanced and prolonged capsaicin-evoked thermal sensitivity. This enhancement occurred with the 5HT dose that produced thermal hyperalgesia, but not with the dose that had no effect. Capsaicin alone evoked thermal hyperalgesia peaking between 5-15 minutes; similar to previous studies (Gilchrist et al., 1996), however, with 5HT pretreatment, the peak thermal sensitivity was prolonged up to 30 minutes. It is unclear whether this is a direct or

indirect effect on TRPV1, but it is clear that peripheral 5HT exacerbates sensitivity to noxious thermal heat. This may be the case with visceral hypersensitivity as well, as it has been reported that 5HT plays a role in enhancing both capsaicin-induced visceral pain (Qin et al., 2010) and capsaicin-evoked currents in mouse colon sensory neurons (Sugiuar et al., 2004).

No studies have examined the effects of anti-serotonergics on capsaicin-evoked thermal hyperalgesia. We found that while neither ketanserin nor granisetron attenuated capsaicin-evoked thermal hyperalgesia, both blocked 5HT-enhancement of capsaicin-evoked thermal hyperalgesia. These results indicate that instead of blocking activation of TRPV1, both anti-serotonergics may be blocking the ability of 5HT to enhance and maintain TRPV1-evoked thermal hyperalgesia. This is important as, during injury 5HT is released peripherally acting on local TRPV1-expressing nociceptors; 5HT may be exacerbating painful conditions via actions at 5HT receptors localized on TRPV1-type nociceptors. This is supported by our previous study illustrating colocalization of 5HT_{2A} and 5HT₃ receptors on TRPV1-expressing sensory neurons (Loyd et al., 2011). We further probed the involvement of the 5HT_{2A} receptors on hyperalgesia with ritanserin, a 5HT_{2A} antagonist with inverse agonist properties. Interestingly, ritanserin induced thermal hyperalgesia with corresponding erythema and enhanced TRPV1-evoked thermal hyperalgesia similar to that observed with 5HT, although more transient. Pretreatment with ketanserin was unable to block the hyperalgesic effects of ritanserin, indicating that ritanserin is not exerting its hyperalgesic effects via the 5HT_{2A} receptor, but rather non-specifically. The current study is limited to examining 5HT modulation of thermal hyperalgesia, however, further pharmacological studies are warranted to understand how ritanserin evokes thermal hyperalgesia and erythema.

As sumatriptan has gained much attention as a potential therapeutic in many pain conditions (Bingham et al., 2001, Kanai et al., 2006, Diamond et al., 2007, Nikai et al., 2008) and has been shown to have both central and peripheral actions (Vera-Portocarrero et al., 2008), we also tested sumatriptan's role in TRPV1-evoked thermal hyperalgesia. Sumatriptan did not significantly attenuate TRPV1-evoked thermal hyperalgesia at 5 minutes, but did reverse hyperalgesia at 10 minutes, indicating that sumatriptan may be reducing the time course of TRPV1-evoked thermal hyperalgesia. This may be occurring via an indirect mechanism on TRPV1 nociceptors which may account for the delay in effect. These data are in concurrence with previous reports that sumatriptan reduces proinflammatory peptide release from sensory neurons (Durham and Russo, 1999, Loyd et al., 2011). Similarly, pretreatment with local sumatriptan reduces capsaicin-evoked neurogenic inflammation in the rat hindpaw, which is blocked by local 5HT_{1B/1D} antagonist (Carmichael et al., 2008). It is interesting that 5HT and sumatriptan, both agonists at the 5HT_{1B/1D} receptor, have opposing actions on thermal hyperalgesia. Our data indicate that 5HT may be evoking hyperalgesia primarily through the excitatory 5HT_{2A} and 5HT₃ receptor pathways, as blocking these receptors attenuates hyperalgesia. On the other hand, sumatriptan is presumably attenuating hyperalgesia via 5HT_{1B/1D} receptors, which are G protein receptors coupled to inhibitory pathways. The results of the present study are limited to illustrating a modulatory effect of 5HT on TRPV1-evoked thermal hyperalgesia, while future studies utilizing 5HT receptor knockouts to examine the role of specific 5HT receptors are warranted.

CONCLUSIONS

From these studies combined with the current literature on the role of 5HT in pain, it can be concluded that 5HT plays a role in enhancing thermal hyperalgesia and that this may occur in the periphery via TRPV1 nociceptors. We provide *in vivo* evidence of an enhancing effect of 5HT on TRPV1-mediated thermal hyperalgesia supporting previous research *in vitro*

reporting sensitization of TRPV1 by 5HT. In addition, our previous *in vitro* studies were conducted on trigeminal sensory neurons, while the present study was conducted in the rat hindpaw innervated by dorsal root ganglia neurons. Future studies utilizing established orofacial pain behavior testing paradigm are warranted to delineate potential differences between the TRPV1 sensory neurons of the trigeminal vs. dorsal root ganglia to better understand trigeminal pain disorders, such as migraine.

Acknowledgments

The authors would like to thank Drs. Kelly Berg and Bill Clarke for helpful discussions during the developmental stage of these experiments. This work was supported by NIH grants DA19585 and NIH, NCRR UL1RR025767 awarded to Dr. Hargreaves, F32 DE021309 awarded to Dr. Loyd and T32 DE14318.

Grant Support: NIH grants DA19585, UL1RR025767 to KMH, F32DE021309 to DRL and 32DE14318

Abbreviations

5HT	serotonin
TRPV1	transient receptor potential V1 channel
PWL	paw withdrawal latency
OVX	ovariectomized
DMSO	dimethyl sulfoxide

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- 5HT's role in peripheral TRPV1-evoked thermal hyperalgesia in the rat hind paw
- Examined changes in thermal sensitivity following peripheral serotonergics treatment
- 5HT pretreatment enhances TRPV1-evoked thermal hyperalgesia in male and female rats
- Attenuated with peripheral pretreatment with ketanserin, graniestron and sumatriptan
- *in vitro* evidence supporting reported enhancing role of 5HT on TRPV1 function *in vitro*

Figure 1.

Peripheral administration of 5HT (0.1 nmol-10 nmol; ipl; n=9-10/group) in a 100 µL volume evokes dose-dependent thermal hyperalgesia in the male rat hindpaw 15 min following injection (A) and increases paw volume as measured by plethysmometry (n=4/group) every 10 minutes for 1 hour (B). * indicates significance at $p < 0.05$ compared to saline vehicle. Dotted line denotes composite basal PWL from all groups.

Figure 2. Peripheral administration of 5HT (0.01 nmol-10 nmol; ipl; n=6-9/group) in a 100 μ L volume evokes dose-dependent thermal hyperalgesia in the hindpaw of intact (A) and ovariectomized (B) female rats. * indicates significance at $p < 0.05$ compared to saline vehicle. Dotted line denotes composite basal PWL from all groups.

Figure 3.

Local pretreatment with the 5HT_{2A} antagonist ketanserin (A) or the 5HT₃ antagonist granisetron (B) 15 minutes prior to 10 nmol/100 μ L 5HT (ipl, n=6-8/group) injection reverses 5HT-evoked thermal hyperalgesia at 15 min post-5HT injection. Also shown is the lack of effect of these drugs on the hyperalgesic paw when injected into the contralateral paw. * indicates significant reversal at $p < 0.05$ compared to saline vehicle. Dotted line denotes composite basal PWL from all groups.

Figure 4.

Change in paw volume as measured by plethysmometry every 10 minutes for an hour following either saline (closed circles), 0.1 nmol/100 μ L ketanserin (open circles) or 0.1 nmol/100 μ L granisetron (open squares) injected ipl 15 minutes prior to 10 nmol/100 μ L 5HT (n=4/group). * indicates significance at $p < 0.05$ compared to saline vehicle. Dotted line denotes percent change in paw volume following saline vehicle injection.

Figure 5.

Effects of 5HT and anti-serotonergics on capsaicin-evoked thermal hyperalgesia (n=6/group). Pretreatment with 5HT (0.1 nmol vs. 10 nmol/ 100 μ l; ipl) 10 minutes prior to 3 nmol/10 μ l capsaicin (A), local pretreatment with the 5HT_{2A} antagonist ketanserin (0.1 nmol/ 100 μ l; grey bars) or the 5HT₃ antagonist granisetron (0.1 nmol/ 100 μ l; black bars) 15 minutes prior to 5HT and capsaicin (B) or ketanserin or granisetron 15 minutes prior to capsaicin alone (C). * indicates significance at $p < 0.05$ compared to saline vehicle. Dotted line denotes composite basal PWL from all groups.

Figure 6.

Peripheral administration of 100 nmol /100 μ l ritanserin (5HT_{2A} antagonist; n=5- 6/group) induces transient thermal hyperalgesia compared to vehicle (A). Also shown is the effect of ritanserin (0.1 vs. 100 nmol/100 μ l) pretreatment on capsaicin-evoked thermal hyperalgesia when given locally 15 minutes prior to 3 nmol/10 μ l capsaicin (B). * indicates significance at $p < 0.05$ compared to saline vehicle. Dotted line denotes composite basal PWL from all groups.

Figure 7.

Effect of local pretreatment with the 5HT_{1B/1D} agonist sumatriptan (0.01-1 nmol/100 μ l; ipl) given 15 minutes prior to capsaicin (3 nmol/10 μ l) with PWL measured every 5 min for 25 min (n=5/group). * indicates significance at p<0.05 compared to saline vehicle. Dotted line denotes composite basal PWL from all groups.

Table 1

Precision analysis of behavioral testing following inhalation anesthesia vs. restraint for injections

Condition	N	Threshold Mean $\pm$ SEM	Coefficient of Variation
<i>Baseline Measure</i>			
Isoflurane	6	10.33 $\pm$ 0.40	0.09
Restraint	6	11.41 $\pm$ 0.29	0.06
<i>15 minutes post-5HT injection</i>			
Isoflurane	6	7.03 $\pm$ 1.43	0.50
Restraint	6	5.99 $\pm$ 0.65	0.23
<i>30 minutes post-5HT injection</i>			
Isoflurane	6	9.88 $\pm$ 1.21	0.30
Restraint	6	11.42 $\pm$ 0.70	0.15