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Abstract

Pain emanating from the female reproductive tract is notoriously difficult to be treated and the prevalence of transient pelvic pain has been placed as high as 70-80% in women surveyed. Although sex hormones, especially estrogen, are thought to underlie enhanced pain perception in females, the underlying molecular and cellular mechanisms are not completely understood. Here we show that the pain-initiating TRPA1 channel is required for pain-related behaviors in a mouse model of estrogen-induced uterine pain in ovariectomized female mice. Surprisingly, 2- and 4-hydroxylated estrogen metabolites (HEMs) in the estrogen hydroxylation pathway, but not estrone, estradiol and 16-HEMs, directly increase nociceptor hyperactivity through TRPA1 and TRPV1 channels, and picomolar concentrations of 2- and 4-hydroxylation estrone (OHE1) can sensitize TRPA1 channel function. Moreover, both TRPA1 and TRPV1 are expressed in uterine-innervating primary nociceptors and their expressions are increased in the estrogen-induced uterine pain model. Importantly, pretreatment of 2- or 4-OHE1 recapitulates estrogen-induced uterine pain-like behaviors and intraplantar injections of 2- and 4-OHE1 directly produce a TRPA1-dependent mechanical hypersensitivity. Our findings demonstrate that TRPA1 is critically involved in estrogen-induced uterine pain-like behaviors, which may provide a potential drug target for treating female reproductive tract pain.

Key words: hydroxylated estrogen metabolites, TRPA1, TRPV1, uterine pain, nociceptor hypersensitivity
Introduction

Chronic pain caused by inflammation and nerve injury is a debilitating and persistent condition without universally effective treatment (1). Pain is a complex and multi-factorial trait with tremendous inter-individual variation (2-5) and females have greater pain sensitivity to many forms of clinical and experimentally-induced pain responses including pressure, heat and chemical irritants (6-8). Clinically, women have higher rates of chronic pain conditions such as migraine, arthritis and fibromyalgia (9-12), and some chronic pelvic pain symptoms only occur in females, such as dysmenorrhea and endometriosis (13).

Sex steroid hormones including progesterone, testosterone and estrogen have been linked to pain perception in both clinical and pre-clinical studies. It was widely accepted that both testosterone and progesterone have anti-pain effects while the effects of estrogen remain controversial (14, 15). For instance, Birgitta et al showed that female-related pain is significantly higher during the menstrual and premenstrual phases when compared to the mid-menstrual and ovulatory phases, which is correlated with the estrogen level (16). In rats, estrogen levels are lower during diestrus (~15–20 pg/mL) and higher during proestrus and estrus (~40–60 pg/mL) (17-19). Fischer et al reported that the nociceptive behavior of female rats in proestrus was significantly lower than that in the diestrus phase (20). On the other hand, numerous studies demonstrated that estrogen promoted pain, for instance, estrogen was reported to exacerbate trigeminal nociceptive pain through up-regulation of TRPV1 and ANO1 channel expression (21). In addition, Ji et al. reported that the visceromotor response fluctuated with menstrual cycle and the intensities of colorectal distensions were significantly higher in proestrus phase where estrogen levels are higher (22). Moreover, chronic estrogen treatment was found to sensitize a subset of high-threshold mechanosensitive afferents innervating the uterine cervix, which might be responsible for
increased pain responses produced by cervical distension (23). However, the molecular and cellular mechanisms underlying estrogen-mediated regulation of primary nociceptor activity remain elusive.

Primary sensory neurons in the dorsal root ganglion (DRG) and trigeminal ganglion (TG) sense noxious stimuli to initiate sensory hypersensitivity such as pain and itch, the warning signs to protect from further tissue damage (24, 25). Transient receptor potential (TRP) channels are molecular sensors in the peripheral sensory nerve endings and contribute to nociceptive transmission by promoting action potential firing and regulating neurotransmitter release (26). Among them, TRPA1 and TRPV1 are selectively expressed by the small size nociceptors that are activated by noxious stimuli to produce either acute or persistent pain (27). Moreover, chronic estrogen treatment increased the excitability of sensory neurons and exacerbated inflammation-induced sensitization of sensory neurons by decreasing rheobase and the threshold of action potentials, thus promoting spontaneous action potential firing (28), suggesting that estrogen might directly regulate nociceptor excitability. However, the molecular basis of estrogen regulation of nociceptor excitability is not understood.

Here we show that although estrogen does not directly affect nociceptor activity, estrogen metabolites in the 2- and 4-hydroxylation pathways promote nociceptor excitability through activation/sensitization of the nociceptive ion channels TRPA1 and TRPV1. Importantly, we show that TRPA1 mediates not only 2- and 4-OHE1-evoked acute pain responses but also visceral hypersensitivity in mouse models of uterine pain induced by either chronic estrogen treatment or acute application of 2- or 4-OHE1. Our studies uncover a previously unrecognized role of TRPA1 in estrogen-related pain perception, advancing the understanding of the role of primary nociceptors in estrogen-induced pain and/or enhancement of pain perception.
Results

Chronic estrogen treatment causes uterine pain

Although clinical evidence has clearly shown that women have higher pain sensitivity compared with men likely due to higher level of estrogen (29, 30), where and how estrogen acts to regulate pain sensitivity in females are not well understood. To address these questions, we generated a mouse model of uterine pain by administrating estradiol benzoate for three consecutive days followed by an intraperitoneal injection of oxytocin at day 4 in ovariectomized wild-type (wt) C57BL/6J female mice implanted with estrogen pellets (Fig 1A). Although it is considered as a mouse model of primary dysmenorrhea (31, 32), this model also resembles human labor pain in which both concentrations of estrogen and oxytocin are elevated (33, 34). As expected, oxytocin-induced uterus contraction markedly increased writhing response in estrogen-treated mice while mice treated with vehicle, estradiol, or oxytocin alone rarely had writhing response (Fig 1B). Of note, in the uterine pain model mice, serum estradiol level was significantly increased to ~150 pg/ml compared with vehicle-treated mice (Fig S1), which is close to the serum estradiol level at the ovulation phase of menstrual cycle in humans.

To quantify pain-like responses in the uterine pain model mice, besides writhing response we also measured voluntary movements including the time mice spent on moving, stationary, and total travelling distance around the cage for the following reasons: (i) monitoring changes in voluntary movements in mice subjected to either inflammation and neuropathy is a simple, observer-independent, and more objective measure of the global level of pain response than reflexive measures, and much more sensitive to analgesic drug effects (35); and (ii) in a mouse model of acetic acid-induced visceral pain model, monitoring changes in movement over time is a more sensitive parameter to identify differences in visceral nociception, compared to writhing reflexes.
Consistent with enhanced writhing response, total traveling distance and time spent on moving were decreased and time spent on stationary was increased in female mice treated with both estradiol benzoate and oxytocin compared to female mice treated with vehicle, estradiol, or oxytocin alone (Fig 1C-F). In contrast, male mice treated with estradiol and oxytocin did not show any visceral pain-like behaviors (Fig S2). Moreover, the pain-inhibiting nonsteroidal anti-inflammatory drug (NSAID), ibuprofen, markedly reduced the numbers of writhing response (Fig 1G). Ibuprofen also increased the total traveling distance and the time spent on moving but decreased the time spent on stationary (Fig 1H-J), confirming that pain-like behaviors were reliably produced in the uterine pain model mice.

**Estrogen metabolites but not estrogens or oxytocin directly activate DRG neurons**

To identify the cellular mechanisms underlying uterine pain response produced by estrogen and oxytocin, we used live-cell Ca\(^{2+}\) imaging to determine whether oxytocin directly activates dissociated DRG neurons. Surprisingly, no effect of oxytocin was observed even with the highest concentration applied (30 µM) (Fig S3A), which is in marked contrast to a recent study showing that 10 µM oxytocin elicited a robust intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)_i]) response in DRG neurons isolated from both male and female wt mice (37). Our result suggests that oxytocin likely causes pain by inducing uterine contractions rather than directly sensitizing nociceptors, which is also supported by the finding that male mice subjected to the same protocol did not develop overt visceral pain responses (Fig S2) (32). Moreover, when 100 µM estrogen including estrone (E1) and estradiol (E2) were applied to DRG neurons, no changes in [Ca\(^{2+}\)_i] were observed (Fig S3B, C). Together, these results suggest that neither oxytocin nor estrogens directly activate and/or sensitize nociceptors.
Notably, estrogen is primarily metabolized through three hydroxylation pathways in which hydroxylation at positions of C2, C4 and C16 converts them into catechol estrogens including 2-hydroxyestrone (2-OHE1), 2-hydroxyestradiol (2-OHE2), 2-methoxyestradiol (2MeOE2), 4-hydroxyestrone (4-OHE1), 4-hydroxyestradiol (4-OHE2), 16-hydroxyestrone (16-OHE1) and estriol (E3) (Fig. S4) (38). Surprisingly, 2-HEMs and 4-HEMs but not E1, E2 or 16-HEMs elicited a robust increase in \([\text{Ca}^{2+}]_i\) in \(\text{wt}\) DRG neurons which also responded to capsaicin (Fig. 2A-C). Interestingly, DRG neurons isolated from both male and female \(\text{wt}\) mice displayed comparable increases in \([\text{Ca}^{2+}]_i\) to both 2- and 4-OHE1 (Fig. 2D), suggesting that there are no sex differences in TRPV1-expressing nociceptor sensitivity to HEMs. 2-OHE1 or 4-OHE1 increased \([\text{Ca}^{2+}]_i\) in DRG neurons in a concentration dependent manner with EC\(_{50}\) values of 1.82 ± 0.10 and 1.24 ± 0.04 \(\mu\text{M}\), respectively (Fig. 2E-H). Consistent with the \(\text{Ca}^{2+}\) imaging results, administration of 100 \(\mu\text{M}\) 2-OHE1 and 4-OHE1 but not E1 also evoked action potential firing and membrane depolarization in \(\text{wt}\) DRG neurons (Fig. 2I-L). These results suggest that 2- and 4-HEMs but not E1, E2 and 16-HEMs directly activate primary nociceptors \textit{in vitro}.

**Estrogen metabolites are a new class of activators for TRPA1 and TRPV1**

We next tested whether estrogen receptors, including nuclear estrogen receptors (ER\(\alpha\) and ER\(\beta\)) and a G protein-coupled estrogen receptor 1 (GPER), mediate 2- and 4-OHE1-induced \([\text{Ca}^{2+}]_i\) responses in DRG neurons by using their specific inhibitors. All of these estrogen receptor inhibitors had no significant effect on the proportions of neurons responding to 2- or 4-OHE1 with \([\text{Ca}^{2+}]_i\) increase (Fig 3A-C), suggesting that 2- and 4-HEM-elicited \([\text{Ca}^{2+}]_i\) responses in DRG neurons are estrogen receptors-independent.
Since TRPA1 is a nociceptive ion channel that is activated by many different classes of chemical compounds, we next tested 2- and 4-OHE1-induced \([\text{Ca}^{2+}]_i\) responses in DRG neurons isolated from the *Trpa1*^{-/-} mice. Strikingly, the proportions of DRG neurons responding to 100 µM 2- and 4-OHE1 were substantially reduced in *Trpa1*^{-/-} DRG neurons when compared to DRG neurons isolated from the *wt* mice (Fig 3D, G, J, and K), suggesting that sensory neuron-expressed TRPA1 is required for 2- and 4-OHE1-induced \([\text{Ca}^{2+}]_i\) responses. However, about 10% of DRG neurons isolated from the *Trpa1*^{-/-} mice could still be activated by 100 µM 2- and 4-OHE1 (Fig 3D, G, J, and K), indicating that TRPA1 is not the sole target for 2- and 4-HEMs in DRG neurons.

Interestingly, all DRG neurons responding to 2- and 4-HEMs could also be activated by the TRPV1 agonist capsaicin (Fig 3A.), we therefore tested whether TRPV1 is also involved. Indeed, the proportions of DRG neurons responding to 100 µM 2- and 4-OHE1 were significantly reduced but not abolished in DRG neurons isolated from the *Trpv1*^{-/-} mice when compared with the *wt* mice (Fig 3E, H, J, and K). Remarkably, 2- and 4-OHE1-induced \([\text{Ca}^{2+}]_i\) responses were completely abolished in DRG neurons isolated from the *Trpa1*^{-/-}/*Trpv1*^{-/-} double knockout (dKO) mice (Fig. 3F, I, J and K), suggesting that both TRPA1 and TRPV1 are involved in \([\text{Ca}^{2+}]_i\) responses induced by 100 µM 2- and 4-OHE1. On the other hand, the \([\text{Ca}^{2+}]_i\) response evoked by a lower concentration of 2- and 4-OHE1 (10 µM) was absent in DRG neurons isolated from the *Trpa1*^{-/-} mice but still present in DRG neurons from the *Trpv1*^{-/-} and *wt* mice (Fig S5), suggesting that 2- and 4-OHE1 activate only TRPA1 but not TRPV1 in primary nociceptors at concentrations lower than 10 µM.

To further confirm that 2- and 4-HEMs directly activate TRPA1 and TRPV1 channels, we heterologously expressed TRP channels in HEK293T cells and measured channel activities using whole-cell patch-clamp recording. Consistent with the results from DRG neurons, both 2- and 4-
HEMs, but not E1, E2 and 16-HEMs, activated TRPA1-dependent whole-cell currents (Fig. 4A-E). 2- and 4-OHE1 activated recombinant TRPA1 channel with EC$_{50}$ values of $0.97 \pm 0.10 \mu$M and $1.07 \pm 0.09 \mu$M, respectively (Fig 4B, D). Moreover, 10 $\mu$M 2- and 4-OHE1 evoked measurable membrane currents in HEK293T cells transfected with TRPV1 but not TRPV2, TRPV3, TRPV4 and TRPM8 channels (Fig. 4F, G). Therefore, both 2- and 4-HEMs constitute a new family of TRPA1 and TRPV1 activators with a much higher potency to TRPA1.

**Structural basis for activation of TRPA1 and TRPV1 by 2-HEMs and 4-HEMs**

Previous studies have shown that electrophiles such as allyl isothiocyanate (AITC) and 4-Hydroxynonenal (HNE) activate TRPA1 through covalent modification of key cysteine and lysine residues within the cytoplasmic domain of the amino-terminal (39, 40). To determine whether 2-OHE1 and 4-OHE1 also activate TRPA1 by interacting with these cysteine and lysine residues, we generated TRPA1-3C (a combination of C621S, C641S, and C665S), TRPA1-K710R and TRPA1-(3C+K710R) mutants that are known to disrupt activation of TRPA1 by known electrophiles using site-directed mutagenesis (41-43). We tested the effect of 2- and 4-HEMs on both wild-type TRPA1 and TRPA1 mutants expressed in HEK293T cells using live-cell Ca$^{2+}$ imaging with a nonreactive TRPA1 agonist, flufenamic acid (FFA), as a positive control (44). We found that HEK293T cells expressing TRPA1-3C or TRPA1-K710R displayed significantly diminished [Ca$^{2+}$]; response induced by either 2-OHE1 or 4-OHE1 when compared with HEK293T cells transfected with wild-type TRPA1 (Fig 5). Strikingly, the 2- and 4-OHE1-elicited [Ca$^{2+}$]; responses were completely abolished in the HEK293T cells expressing TRPA1-(3C+K710R) mutant (Fig 5), suggesting that 2-OHE1 and 4-OHE1 directly activate TRPA1 through covalent modifications of key cysteine and lysine residues.
Many TRPV1 activators including capsaicin and resiniferatoxin activate TRPV1 channel by interacting with the “vanilloid-binding pocket” in the cytosolic side, especially Y512 and S513 that specify sensitivity to vanilloid ligands (45-47). We thus examined whether these two vanilloid-sensitive TRPV1 residues are involved in the activation of TRPV1 by 2- and 4-OHE1. Indeed, 2- and 4-OHE1-induced [Ca\(^{2+}\)]\(_i\) responses were markedly reduced in the Y512A mutant and almost completely abolished in the S513Y mutant but not M548L or T551I (Fig S6). These results demonstrate that the “vanilloid-binding pocket” is essential to the interaction between 2- and 4-HEMs and TRPV1.

**Picomolar concentrations of 2- or 4-OHE1 potentiate AITC- but not Cap-induced [Ca\(^{2+}\)]\(_i\) response in DRG neurons**

Sensitization of TRPA1 and TRPV1 is an important mechanism underlying sensory hypersensitivity (48, 49). Therefore, we next tested if pre-applied physiologically relevant concentrations of 2- or 4-OHE1 could sensitize TRPA1 and TRPV1 in DRG neurons. Strikingly, pre- and co-application of 10 to 100 pM 2- and 4-OHE1 but not E1 or 16-OHE1 (which were ineffective even at 1 μM) concentration-dependently increased [Ca\(^{2+}\)]\(_i\) responses elicited by 5 μM AITC which otherwise elicited a small [Ca\(^{2+}\)]\(_i\) response when applied alone (Fig 6 and S7). In marked contrast, pre-treatment of 2- or 4-OHE1 didn’t increase [Ca\(^{2+}\)]\(_i\) response induced by capsaicin (Fig S8). Combined, these results suggest that 2- and 4-OHE1 sensitize TRPA1 function in primary nociceptors at picomolar concentrations besides direct activation of TRPA1 at higher concentrations.

**TRPA1 and TRPV1 are expressed in uterine-innervating DRG neurons**
To determine whether DRG neurons innervating uterus can be activated by HEMs, we injected CTB647 into the uterine wall of PirrGCaMP3 mice (Fig 7A) and isolated DRG neurons 5 days after injections. We did Ca²⁺ imaging and found 2-OHE1 could also activate CTB647-labeled DRG neurons (Fig 7B-D). Since prior studies showed increased expression of TRP channels by estrogen treatment (50), we next tested whether chronic treatment of estradiol in uterine pain model alters the expression of TRPA1 and TRPV1 using single cell qRT-PCR on CTB488-labeled DRG neurons (Fig 7E, F). The results showed that in control mice (Vehicle + Oxytocin), TRPA1 mRNA transcripts were detected in 65.6% (21 of 32) and TRPV1 mRNA transcripts were detected in 84.4% (27 of 32) of CTB488-labeled DRG neurons (Fig. 7F). 90.5% (19 of 21) of the TRPA1-positive CTB488-labeled DRG neurons also expressed TRPV1 mRNA transcripts. In uterine pain model mice, TRPA1 mRNA transcripts were detected in 72.4% (21 of 29) and TRPV1 mRNA transcripts were detected in 82.8% (24 of 29) of CTB488-labeled DRG neurons (Fig. 7F) (Estradiol + Oxytocin). There was also 90.5% (19 of 21) of the TRPA1-positive CTB488-labeled DRG neurons that expressed TRPV1 mRNA transcripts. More importantly, both mRNA transcript levels of mTRPA1 and TRPV1 were significantly increased in uterus-innervating DRG neurons isolated from uterine pain model mice when compared to those from control mice (Fig 7G, H).

**TRPA1 mediates 2-OHE1- and 4-OHE1-induced acute mechanical nociception**

We next tested if estrogen metabolites can directly produce acute nociception through activating TRPA1. Indeed, intraplantar injections of 2-OHE1 and 4-OHE1 but not E1 or 16-OHE1 provoked a robust mechanical pain response in a dose-dependent manner (Fig 8A, B and Fig S9), which lasted for more than 4 hrs. Furthermore, mechanical allodynia, induced by intraplantar injections of 2-OHE1 or 4-OHE1, was significantly reduced by both pharmacological inhibition and genetic ablation of TRPA1 but not TRPV1 function (Fig 8C-F), suggesting that acute applications of 2-
and 4-HEMs directly evoke mechanical hypersensitivity in a TRPA1-dependent but not TRPV1-dependent manner.

**TRPA1 mediates uterine pain caused by chronic estrogen treatment**

We showed that TRPA1 is directly activated/sensitized by 2- and 4-HEMs and mediates acute nociception evoked by 2- and 4-OHE1 and previous studies also demonstrated that nociceptor-expressed TRPA1 and TRPV1 mediate both neurogenic inflammation and pain (24, 51). We therefore investigated if TRPA1 and/or TRPV1 channels are involved in the mouse model of uterine pain induced by chronic estrogen treatment. We measured writhing responses and voluntary movements in the wt C57BL/6J mice and their congenic Trpa1<sup>−/−</sup> and Trpv1<sup>−/−</sup> mice (Fig 9A-E). The total moving distance and the total time spent on moving were significantly increased while the number of writhing responses and the time spent on stationary were significantly decreased in the Trpa1<sup>−/−</sup> but not in the Trpv1<sup>−/−</sup> mice when compared with the wt mice (Fig 9), suggesting that TRPA1 but not TRPV1 is required for pain-related responses in this mouse model of uterine pain. We also generated nociceptor-specific Trpa1 conditional knockout mice by crossing Trpv1<sup>Cre</sup> mice with Trpa1<sup>f/f</sup> mice. To confirm the successful ablation of TRPA1 from TRPV1 positive nociceptors, we isolated DRG neurons from both Trpa1<sup>f/f</sup> control littermates and Trpv1<sup>Cre::Trpa1<sup>f/f</sup></sup> mice and tested the effect of 2-OHE1 on isolated DRG neurons (Fig 10A-C). 2-OHE1 evoked [Ca<sup>2+</sup>]<i>i</i> response was significantly reduced in DRG neurons isolated from Trpv1<sup>Cre::Trpa1<sup>f/f</sup></sup> mice compared to those isolated from Trpa1<sup>f/f</sup> control littermates (Fig 10C). More importantly, in this mouse model of uterine pain, Trpv1<sup>Cre::Trpa1<sup>f/f</sup></sup> mice showed significantly reduced pain-related behaviors when compared to Trpa1<sup>f/f</sup> control littermates, recapitulating the phenotype of the global TRPA1 knockout mice (Fig 10D-H). Taken together,
these results demonstrated the important role of nociceptor-expressed TRPA1 in the generating of estrogen-induced uterine pain.

**Acute applications of 2-OHE1 and 4-OHE1 elicit uterine pain through TRPA1.**

We showed that 2- and 4-HEMs directly activate TRPA1 channel at high concentrations and sensitize TRPA1 function at picomolar concentrations. More importantly, acute application of 2-OHE1- and 4-OHE1 could elicit acute mechanical nociception in a TRPA1 dependent-manner, suggesting that 2- and 4-HEMs may be the cause of the visceral pain behavior in the mouse model of uterine pain generated by chronic estrogen treatment. To test this possibility, we modified the protocol of the uterine pain model and treated the mice acutely with 2- and 4-OHE1 for 30 min instead of treating mice with estrogen for three days. Strikingly, acute treatment of 2- and 4-OHE1 but not E1 or 16-OHE1 for 30 min followed by oxytocin application induced a robust writhing response, which was significantly diminished in the *Trpa1*−/− but not *Trpv1*−/− mice (Fig 11A-E and Fig S10). The decreased total moving distance and time spent on moving and increased time spent on stationary after treatment of 2- and 4-OHE1 followed by oxytocin application were also reversed in the *Trpa1*−/− but not *Trpv1*−/− mice (Fig 11 B-H), suggesting acute application of 2- or 4-OHE1 is sufficient to sensitize TRPA1 and cause uterine pain in female mice.

**Discussion**

A thorough understanding of the cellular and molecular mechanisms accounting for pain processing by estrogen is critical because it offers an opportunity to provide pain relief and personalized pain therapy for estrogen-related pain. In this study, we show that nociceptor-expressing TRPA1 is critically involved in pain-related behaviors in a mouse model of uterine pain,
potentially through 2- and 4-HEMs-mediated sensitization mechanism. First, both live-cell Ca\textsuperscript{2+} imaging and whole-cell patch-clamp recordings show that 2- and 4-HEMs directly activate DRG neurons through TRPA1 and TRPV1 channels in a concentration-dependent manner. Second, at picomolar concentrations, 2- and 4-OHE1 potentiate TRPA1 but not TRPV1 function. Third, intraplantar injections of 2- and 4-OHE1, but not estrogen, evoke acute pain responses \textit{in vivo} in a TRPA1-dependent manner. Lastly, acute application of 2- and 4-OHE1 is sufficient to produce uterine pain in female mice through TRPA1. Our findings provide convincing evidence that estrogen metabolites can directly activate/sensitize the TRPA1-expressing nociceptors, advancing the understanding of the role of primary nociceptors in estrogen-induced pain and/or enhancement of pain perception in females.

Prior studies have demonstrated that TRP channel expression is regulated by estrogen signaling at both transcriptional and translational levels. For instance, exogenously applied estrogen can regulate the expression of several nociceptive ion channels including TRPA1 and TRPV1 in both nociceptors and non-neuronal cells (21, 52-56). Corroborating with these findings, our retrograde labelling experiments also showed that the uterus-innervating nociceptors express both TRPA1 and TRPV1 and both expression levels are increased in the mouse model of uterine pain induced by chronic estrogen treatment. Our behavioral results support the dominant role of nociceptor-expressed TRPA1 in mediating the estrogen-induced uterine pain-like behaviors as genetic ablation of TRPA1 function in TRPV1-lineage neurons markedly reduced the estrogen-induced responses and recapitulates the phenotype seen in the global TRPA1 knockout mice.

Surprisingly, we showed that estrogen metabolites but not estrogen itself directly activate TRPA1 and TRPV1 channels and promote nociceptor excitability. Consistent with our findings, Ma et al also reported that catechol estrogens stimulate insulin secretion in pancreatic β-cells through
TRPA1 activation by HEMs at micromolar concentrations (57). Interestingly, although both TRPA1 and TRPV1 are direct targets of 2- and 4-HEMs, only TRPA1 mediates the visceral hypersensitivity caused by estrogen-induced sensitization in the mouse model of uterine pain and acute mechanical nociception evoked by either 2- or 4-OHE1. This discrepancy may result from the lower efficacy and efficiency of HEM activation of TRPV1, as shown in our whole-cell patch-clamp studies using TRPV1-expressing HEK293T cells.

Furthermore, 2- and 4-HEMs at picomolar concentrations only sensitize TRPA1 but not TRPV1 channels. It should be noted that the peak concentration of estradiol in blood reaches up to more than 500 pM during the human menstrual cycle and in the mouse uterine pain model (Fig S1) (58). Although the estrogen metabolites derived from estradiol at such low concentrations should not effectively activate TRPA1, our results showed that 10 pM 2- and 4-HEMs pretreatment is sufficient to potentiate AITC-induced [Ca^{2+}]_i increases in DRG neurons, suggesting that low concentrations of 2- and 4-HEMs in the physiological range might be able to sensitize TRPA1 function in primary nociceptors in vivo.

Although recent exciting studies have significantly advanced our understanding about the importance of estrogen signaling in the CNS and immune system in pain perception (59-66), our studies reveal novel functions of TRPA1 channel and TRPA1-expressing primary nociceptors in mediating the excitatory actions elicited by 2- and 4-HEMs, suggesting additional molecular and cellular targets for estrogen metabolites in the peripheral nervous system. Identification of primary nociceptors as a critical player in estrogen-related pain sensitivity will fill a critical void in understanding how nociceptive TRP channels, especially TRPA1, detect estrogen metabolism and translate it into increased sensory hypersensitivity. These studies should also offer new insights into the development of effective and safe personalized medicines for estrogen-related pain.
perception. Specifically, rationale-based therapies can be developed to reduce female-dominant pain by targeting the TRPA1 signaling, which should avoid the deleterious side effects of current pain medicines. Furthermore, blocking TRPA1 to attenuate estrogen-induced pain sensitization can diminish unwanted irritating side effects produced by estrogen replacement therapy without compromising the beneficial effects mediated by nuclear estrogen receptor signaling.

**Material and method**

**Mice**

Adult female *C57BL/6J* mice used in this study were purchased from Jackson Laboratories (Bar Harbor, ME, USA). *Trpa1*+/+ and congenic *Trpa1*−/− mice, *Trpv1*+/+ and congenic *Trpv1*−/− mice on the *C57BL/6J* background were also obtained from Jackson Laboratory (Bar Harbor, ME, USA). Both *Trpv1*−/− and *Trpa1*−/− mice were continuously backcrossed to *C57BL/6J* mice for more than 10 generations. *Trpv1*−/− and *Trpa1*−/− mice were crossed to generate *Trpa1*−/−/*Trpv1*−/− double KO mice. Body weight- and gender-matched wt, *Trpa1*−/−, *Trpv1*−/− and *Trpa1*−/−*Trpv1*−/− double KO mice were used at about 10-weeks old for all the experiments. *Trpv1*Cre mice were donated by Dr. Mark Hoon from NIH (Bethesda, USA) and *Trpa1*floxed mice were kindly provided by Dr. Scott Earley from University of Nevada (Reno, USA). *PirtGCaMP3* mice were generous gifts from Dr. Xinzhong Dong of Johns Hopkins University (Baltimore, USA). All mice were bred at Washington University in Saint Louis, School of Medicine, and housed under a 12 h light/12 h dark cycle with food and water provided. All mice were randomly allocated to different experimental groups and all behavior tests were done by someone who was blinded to the experimental design.

**CTB injection**
Adult female $Pir^{GCaMP3}$ or $C57BL/6J$ mice were anesthetized with 4% isoflurane followed by 1.5% isoflurane to maintain anesthesia and artificial tear was used to prevent dehydration of eyes. Mice were placed on a sterile surgical pad and covered with a sterile surgical drape after shaving and sterilization of the abdomen. To expose the uterus, a midline incision was made through the abdominal wall and 2 injections of CTB647 or CTB488 was injected bilaterally into the wall of each uterine horn at 100 nL/minute with a pulled glass pipette. After injection, the abdominal wall was sutured, and skin was closed with surgical sutures and antibiotic ointment was applied to the surgical site. After 5 days postoperatively, tissues were collected for analysis.

**Isolation and culture of DRG neurons**

Mouse DRG neurons were isolated and cultured as previously described (67, 68). According to published retrograde tracing results from uterus, DRG between T12 and S2 were isolated (10.3389/fnins.2014.00202). Briefly, the spinal column was firstly removed from mouse and then placed in 2 ml ice-cold Hank’s Balanced Salt Solution (HBSS). Laminectomies were performed and bilateral T12-S2 DRG were dissected out. Connective tissues were removed and DRG were placed to 1 ml Ca$^{2+}$/Mg$^{2+}$-free HBSS with 2 μl saturated NaHCO$_3$, 0.35 mg L-cysteine, and 20 U papain (Worthington Biochemical, Lakewood, NJ, USA) and incubated at 37°C for 10 minutes. After centrifugation, the supernatants were removed and DRG was further treated with 1 mL Ca$^{2+}$/Mg$^{2+}$-free HBSS containing 3.75 mg collagenase type II (Worthington Biochemical, Lakewood, NJ, USA) and 7.5 mg dispase (Worthington Biochemical) for 15 min. Neurons were gently triturated, pelleted, and then resuspended in Neurobasal-A culture medium containing 2% B-27 supplement (ThermoFisher Scientific, Waltham, MA, USA), 100 U/mL penicillin plus 100 µg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA), 100 ng/mL nerve growth factor (NGF, Sigma), 20 µg/mL glial cell-derived neurotrophic factor (GDNF, Sigma-Aldrich) and 10%
heat-inactivated FBS (Sigma-Aldrich). After plating, DRG neurons were kept in a humidified incubator at 37 °C for at least 24 h.

**Single DRG neuron picking**

DRG neurons were purified with a 15% BSA density gradient column and CTB488-labeled neurons were visually identified using a Nikon Eclipse TE200-S microscope (Tokyo, Japan). The identified neuron was picked using a micromanipulator (Sutter Instrument, Novato, CA). A pulled glass electrode filled with HBSS solution was used to get individual DRG neurons and then the neuron was drawn into the tip of glass electrode using negative pressure. The neuron was transferred to a PCR tube containing 10 µL of Single Cell Lysis/Dnase I solution (4458237, Invitrogen) and was processed according to the manual.

**Single cell qRT-PCR**

Single cell qRT-PCR were performed with Invitrogen Single Cell-to-CT™ Kit (4458237, Invitrogen) according to the manufacturer’s manual. TaqMan assays were used to measure abundance of Trpa1 (TaqMan Assay ID: Mm01227437_m1), Trpv1 (TaqMan Assay ID: Mm01246300_m1), and Gapdh (TaqMan Assay ID: Hs02758991_g1). HEK293T cell culture and transfection

HEK293T cells were purchased from ATCC (CRL-3216). Before culturing in lab, Cells were tested for mycoplasma contamination. Cells were cultured in DMEM (Life Technologies, Carlsbad, CA, USA) containing 100 U/mL penicillin plus 100 µg/mL streptomycin and 10% FBS (Life Technologies) in a humidified incubator at 37 °C with 5% CO2.

cDNAs for mouse TRPV1 (mTRPV1), individual mTRPV1 mutants, human TRPA1 (hTRPA1), individual hTRPA1 mutants, mouse TRPV2 (mTRPV2), mouse TRPV3 (mTRPV3), rat TRPV4
(rTRPV4), or mouse TRPM8 (mTRPM8) were transiently transfected to HEK293 cells for at least 24 h using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). QuikChange II XL Mutagenesis Kit (Agilent Technologies, Inc., Santa Clara, CA, USA) and DNA sequencing were performed to make all the mutants. TRPA1 and TRPV1 mutants were used in published paper (10.1038/s41467-017-01056-8).

**Ratiometric measurement of intracellular free Ca$$^{2+}$$**

Before calcium imaging experiments, cultured DRG neurons and HEK293T cells transfected with TRP channels, or their mutant plasmid were placed in culture medium containing 4 µM Fura-2 AM (Life Technologies) at 37°C for 60 minutes. Then cells were washed 3 times and placed in HBSS at room temperature for 30 minutes. During the experiment, Fluorescence at 340 nm and 380 nm excitation wavelengths was recorded using an inverted Nikon Ti-E microscope controlled by NIS-Elements imaging software (Nikon Instruments, Inc., Melville, NY, USA). Fura-2 ratios (F340/F380) was used to reflect changes in [Ca$$^{2+}$$]$$\text{i}$$ upon stimulation and threshold of activation was defined as 3 SD above the average (~20% above the baseline).

**Whole-cell patch-clamp recordings**

Whole-cell patch-clamp recordings were done by an Axon 700B amplifier (Molecular Devices, Sunnyvale, CA, USA) at room temperature (22–24°C) on the stage of an inverted phase-contrast microscope equipped with a filter set for GFP visualization (67, 68). For DRG neurons, small or medium diameter cells (<25 µm diameter) were selected in recording. For transfected HEK293T cells, GFP-expressing cells were selected during recording. Pipettes were pulled using a Sutter P-1000 pipette puller from borosilicate glass (BF 150-86-10; Sutter Instrument, Novato, CA, USA). The pulled pipettes had resistance of 2-5 megaohms. The pipette solution contains 140 mM KCl,
2 mM EGTA, and 10 mM HEPES and pH was adjusted to 7.3 with KOH, and osmolarity was adjusted to 315 mOsm/l with sucrose. Extracellular solution contains 140 mM NaCl, 5 mM KCl, 0.5 mM EGTA, 1 mM MgCl2, 10 mM glucose, and 10 mM HEPES (pH was adjusted to 7.4 with NaOH, and the osmolarity was adjusted to ≈340 mOsm/l with sucrose).

**Ovariectomy and 17β-estradiol replacement and assay for 17β-estradiol**

Female mice were ovariectomized bilaterally and a slow-release pellet of 17β-estradiol (0.1 mg/pellet, 90-day release, Innovative Research of America, Sarasota, FL) was inserted subcutaneously. Serum concentrations of 17β-estradiol was measured at the Ligand Assay and Analysis Core in the Center for Research in Reproduction at the University of Virginia (Charlottesville, VA) using ELISA kits. The assay sensitivity was 3 pg/ml.

**Mouse model of uterine pain and analysis of voluntary movements**

For uterine pain mouse model, the ovariectomized and 17β-estradiol replaced female mice were pretreated with estradiol benzoate (1 mg/kg/day) intraperitoneally for 3 consecutive days. On the fourth day, mice were intraperitoneally injected with 0.4 U of oxytocin. Immediately after oxytocin injection, the mice were unrestrainedly placed in a 25 cm × 25 cm customed cage and the voluntary movement of the mice were recorded for 30 min. The number of writhing responses within 30 min was counted. The distance mice travelled around the cage, the time mice spent on moving, and the time mice spent on stationary within the first 5 minutes were measured using the Ethovision XT tracking software (Noldus). After the recording, the animals were used to test mechanical allodynia.

**Mechanical allodynia behavioral test**

Mechanical allodynia test was performed as previous described (69, 70). Briefly, each mouse was placed individually in a Plexiglas chamber and acclimated for 1 hour before testing. Paw
withdrawal threshold was measured using the Von Frey filaments, starting with the 0.4 g filament. Von Frey filaments ranging from 0.02 g to 2 g bending force were applied to the plantar skin of the right hind paw, using the up down method to determine the threshold. After baseline determination, 10 μl of vehicle or estrogen metabolites were injected intraplanarly in the right hind paw of mice (ipsilateral paw). The mice were then tested for mechanical allodynia over a time course of 12 h (0.5, 1, 2, 4, 8, and 12 h). AMG9810 (10 mg/kg, i.p. injection) and A967079 (10 mg/kg, i.p. injection) were given 30 minutes before paw injections of 2-OHE1 or 4-OHE1. All experiments were performed blind with respect to genotype and treatment.

Statistics Analysis

All retrograde tracing, patch-clamp, and Ca\(^{2+}\) imaging experiments were repeated using DRG neurons from at least three different mice. GraphPad Prism 7.0 (GraphPad) was used to perform statistical analysis. Unless stated otherwise, data are presented as mean ± standard error of the mean (SEM). In general, the exact value of the sample size (n) is presented in the figure legends. The unpaired two-tailed student’s t test with equal variance was used where two groups were compared. One-way Analysis of variance (ANOVA) or two-way ANOVA with Bonferroni post-hoc analysis was used to calculated P values where multiple groups were compared, as figure legends showed. Significance is labeled as: ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05, n.s., not significant. P < 0.05 was considered significantly different.

Study approval

All the animal experimental procedures were done according to the guidelines of the International Association for the Study of Pain and the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of Washington University School of Medicine.
Author contributions
The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. Z.X., J.F., T.C. and H.H. designed the experiments. Z.X., J.F. and T.C. performed the experiments and analyzed the data. R.M., Y.W., F.L. and S.K.E. assisted with ovariectomy and estrogen pellet implantation. M.D.E. and A.D. helped in estrogen detection. Z.Y., Y.Z., K.Z., Y.Y., X.H. assisted with behavior assays. Q.L donated mice. Z.X., J.F. and H.H. wrote the manuscript. H.H. supervised the project. First authorship order position was listed based on intellectual contribution to design of the study and interpretation of data.

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Figures and figure legends

Fig 1. Pain-related responses in the mouse model of uterine pain. (A) Schematic diagram illustrates the protocol for generating a mouse model of uterine pain in ovariectomized female mice implanted with estrogen pellets. (B) Oxytocin-induced writhing response in “Vehicle” group, “Estradiol” group (pretreatment of estradiol benzoate without application of oxytocin), “Oxytocin”
group (application of oxytocin without pretreatment of estradiol benzoate) and “Estradiol+Oxytocin” group (pretreatment of estradiol benzoate followed by application of oxytocin). n=6 mice per group, One-way ANOVA. ****p < 0.0001. (C) Representative images illustrate heat maps of voluntary movements of mice subjected to different treatments in open field test in home cages. (D-F) Quantification of voluntary movements including total moving distance (D), total time spent on moving (E) and total time spent on stationary (F), n=6 mice per group, One-way ANOVA. ***p < 0.001, ****p < 0.0001. (G-J) Ibuprofen significantly reduces the number of writhing response (G) and total time spent on stationary (J) while increases the total moving distance (H) and total time spent on moving (I), n=6 mice per group, unpaired student’s t-test. **p < 0.01. All data are expressed as means ± S.E.
Fig 2. Estrogen metabolites 2- and 4-HEMs directly activate DRG neurons. (A-B) Representative time-lapse traces of 2-OHE1 (A) and 4-OHE1-induced (B) [Ca$^{2+}$]$_i$ responses in DRG neurons from wt mouse. (C) Percentage of DRG neurons responding to estrogen and its metabolites. n=5 mice (>200 neurons each). (D) Percentage of DRG neurons isolated from female and male mice responding to 2- and 4-OHE1. n= 5 to 6 coverslips per group from 4 mice (>150 neurons each). (E-H) Representative [Ca$^{2+}$]$_i$ responses and dose-response curves of [Ca$^{2+}$]$_i$ responses in DRG neurons from wt mouse responding to different concentrations of 2-OHE1 (E and F) and 4-OHE1 (G and H). (I-K) Representative voltage traces show membrane potential depolarization and action potential firing in DRG neurons isolated from wt mice induced by E1 (I), 2-OHE1 (J) and 4-OHE1 (K). (L) Quantification of E1, 2-OHE1, 4-OHE1, and AITC-induced membrane potential depolarization in DRG neurons isolated from wt mice. n=4 to 5 from 3 mice.
Fig 3. TRPA1 and TRPV1 are required for 2- and 4-OHE1-induced DRG activation. (A) Representative traces show that the GPER antagonist G15 have no effect on 2-OHE1-induced [Ca^{2+}]_i responses in DRG neurons. (B-C) Summarized data show that estrogen receptor antagonists (at 1 μM) have no effect on 2-OHE1-induced [Ca^{2+}]_i responses in DRG neurons. n=5 coverslips from 3 mice per group (>150 neurons each). One-way ANOVA, n.s. not significant. (D–I) Representative time-lapse traces of 2-OHE1- (D-F) and 4-OHE1-induced (G-I) [Ca^{2+}]_i responses
in the *Trpa1*<sup>−/−</sup>, *Trpv1*<sup>−/−</sup>, *Trpa1*<sup>−/−</sup>*Trpv1*<sup>−/−</sup> DRG neurons. (J-K) Percentage of DRG neurons responding to 2-OHE1 (J) and 4-OHE1 (K) in DRG neurons isolated from *wt, Trpa1*<sup>−/−</sup>, *Trpv1*<sup>−/−</sup> and *Trpa1*<sup>−/−</sup>*Trpv1*<sup>−/−</sup> mice. n=5 coverslips from 4 mice per group (>200 neurons each). **p<0.01, ***p < 0.001, ****p < 0.0001, One-way ANOVA.
Fig 4. 2- and 4-HEMs activate recombinant TRPA1 and TRPV1 expressed in HEK293T cells.

(A) Representative current traces (left) and I–V curves (right) of TRPA1-dependent currents activated by 2-OHE1 (10 μM) and AITC (100 μM). (B) Concentration-current density curve of 2-OHE1-activated membrane current in TRPA1-expressing HEK293T cells. The EC50=0.97±0.10 μM. n=5. (C) Representative current traces (left) and I–V curves (right) of TRPA1-dependent currents induced by 10 μM 4-OHE1 and 100 μM AITC. (D) Concentration-current density curve of 4-OHE1-induced membrane current in TRPA1-expressing HEK293T cells. The EC50=1.07 ± 0.09 μM. n=5. (E) Bar charts illustrate whole-cell current densities produced by estrogen and its metabolites in TRPA1-expressing HEK293T cells. All chemicals were applied at 10 μM. n=3 to 6. (F-G) Whole-cell current densities produced by 10 μM 2-OHE1 (F) and 10 μM 4-OHE1 (G) in
HEK293T cells transfected with TRPV1, TRPV2, TRPV3, TRPV4 and TRPM8 channels. n=3 to 4 from three independent experiments.
Fig 5. Structural basis of 2-OHE1- and 4-OHE1-induced TRPA1 activation. (A-D) Averaged time-lapse traces of 2-OHE1-elicited [Ca^{2+}]_{i} responses in HEK293T cells transfected with TRPA1 (A), TRPA1-3C (B), TRPA1-K710R (C) and TRPA1-(3C+K) (D) constructs, FFA was used as a positive control; (E) Quantification of 2-OHE1-induced [Ca^{2+}]_{i} responses in HEK293T cells transfected with TRPA1 and its mutants. (F-I) Averaged time-lapse traces of 4-OHE1-induced [Ca^{2+}]_{i} responses in HEK293T cells transfected with TRPA1 (F), TRPA1-3C (G), TRPA1-K710R (H) and TRPA1-(3C+K) (I) constructs; J. Quantification of 4-OHE1-induced [Ca^{2+}]_{i} responses in
HEK293T cells transfected with TRPA1 and its mutants. **p < 0.01, ****p < 0.0001, One-way ANOVA, n=3 to 6 coverslips per group (>100 cells each) from three independent experiments.
Fig 6. 2-OHE1- and 4-OHE1 potentiate AITC-induced \([\text{Ca}^{2+}]_i\) responses in DRG neurons. (A-B) Pre-application of 100 pM 2-OHE1 (B) but not vehicle (A) for 10 min potentiates 5 μM AITC-induced \([\text{Ca}^{2+}]_i\) responses in DRG neurons isolated from wt mice. (C) Bar charts show the potentiating effect of 2-OHE1 on 5 μM AITC-induced \([\text{Ca}^{2+}]_i\) responses in a dose-dependent manner. (D-E) Effects of vehicle (D) and 100 pM 4-OHE1 (E) on 5 μM AITC-induced \([\text{Ca}^{2+}]_i\) responses in DRG neurons isolated from wt mice. (F) Bar charts illustrate the potentiating effect of 4-OHE1 on 5 μM AITC-induced \([\text{Ca}^{2+}]_i\) responses in a dose-dependent manner. *p<0.05, **p<0.01, ****p<0.0001, One-way ANOVA, n=3-6 coverslips from at least 3 mice (>200 neurons each).
Fig 7. **TRPA1 and TRPV1 are expressed in uterine-innervated nociceptors.** (A) Schematic representation of intra-uterine injections of CTB647 into the *Pir tgCaMP3* mice. (B) Representative fluorescence images of cultured DRG neurons show that 2-OHE1 and AITC evoked [Ca^{2+}]_i responses in a CTB647-labeled neuron. Scale bar=50 μm. (C) Representative time-lapse trace of 2-OHE1-induced [Ca^{2+}]_i response in the CTB647-labeled DRG neuron. (D) Percentage of CTB647-labeled DRG neurons responding to 2-OHE1, AITC, and Cap. n=5 coverslips from 3
mice. (E) Schematic representation of intra-uterine injections of CTB488 into the C57BL/6J mice. (F) Donut plots showing expression and co-expression of TRPA1 and TRPV1 in 32 individual retrogradely traced colon-innervating DRG neurons from control mice (left) and in 29 individual retrogradely traced colon-innervating DRG neurons from uterine pain mice (right). n=32 cells from 4 mice for control group and n=29 cells from 4 mice for uterine pain group. (G) Statistic data of single cell qRT-PCR in CTB647-label neurons show the increased expression of TRPA1 in uterine pain model mice compared to control mice. Unpaired t-test, *P<0.05. n=21 cells from 4 mice for “Vehicle + Oxytocin” group and n=21 cells from 4 mice for “Estradiol + Oxytocin” group. (H) Statistic data of single cell qRT-PCR in CTB647-label neurons show the increased expression of TRPV1 in uterine pain model mice compared to control mice. Unpaired t-test, **P<0.01. n=27 cells from 4 mice for “Vehicle + Oxytocin” group and n=24 cells from 4 mice for “Estradiol + Oxytocin” group.
Fig 8. Intraplantar injections of 2-OHE1 and 4-OHE1 produce TRPA1-dependent mechanical allodynia in ovariectomized female mice implanted with estrogen pellets. (A-B) Intraplantar administration of 2-OHE1 (A) and 4-OHE1 (B) induced mechanical allodynia in the ovariectomized wt female mice implanted with estrogen pellets in a dose-dependent manner. *p < 0.05, ***p < 0.001, ****p < 0.0001, Two-way ANOVA, n=5. (C-D) TRPA1 specific
inhibitor A967079 but not TRPV1 specific inhibitor AMG9810 reverts mechanical allodynia induced by 3 nmol 2-OHE1 (C) or 4-OHE1 (D). $p < 0.05$, $$p < 0.01$, $$$p < 0.001$, Two-way ANOVA, n=5. (E-F) Mechanical allodynia induced by 3 nmol 2-OHE1 (E) and 4-OHE1 (F) is markedly reduced in Trpa1⁻/⁻ but not Trpv1⁻/⁻ mice. ##$p < 0.01$, ###$p < 0.001$, ####$p < 0.0001$, Two-way ANOVA with Bonferroni post-hoc analysis, n=5.
Fig 9. Genetic ablation of TRPA1 but not TRPV1 significantly inhibits pain-related behaviors in a mouse model of uterine pain induced by estrogen in ovariectomized female mice implanted with estrogen pellets. (A) Representative images illustrate heat maps of voluntary movements of wt, Trpv1−/− and Trpa1−/− mice subjected to 3 days of estrogen treatment followed by oxytocin application. (B-E) Statistic data show the number of writhing responses (B), total moving distance (C), total time spent on moving (D) and total time spent on stationary (E) in wt, Trpa1−/− and Trpv1−/− mice subjected to 3 days of estrogen treatment followed by oxytocin application. ** p<0.01, *** p<0.001, n=6, One-way ANOVA.
Fig 10. Conditional knockout TRPA1 from TRPV1-positive nociceptors significantly inhibits pain-related behaviors in a mouse model of uterine pain. (A-B) Representative time-lapse trace of 2-OHE1-induced [Ca^{2+}]_{i} response in DRG neurons isolated from Trpa1^{f/f} mice (A) and Trpv1^{Cre::Trpa1^{f/f}} mice (B). (C) Percentage of DRG neurons responding to 2-OHE1 in DRG neurons isolated from Trpa1^{f/f} mice and Trpv1^{Cre::Trpa1^{f/f}} mice. n=5 coverslips from 3 mice per group. ****P<0.0001, unpaired t-test. (D) Representative images illustrate heat maps of voluntary movements of Trpa1^{f/f} (left) and Trpv1^{Cre::Trpa1^{f/f}} mice (right) subjected to 3 days of estrogen treatment followed by treatment of oxytocin. (E-H) Statistic data show the number of writhing
responses (E), total moving distance (F), total time spent on moving (G) and total time spend on stationary (H) in $Trpa1^{f/f}$ and $Trpv1^{Cre:Trpa1^{f/f}}$ mice subjected to 3 days of estrogen treatment followed by treatment of oxytocin. n=8 mice per group. **P<0.01, ***P<0.001, unpaired t-test.
Fig 11. Acute administration of 3 nmol 2- or 4-OHE1 for 30 min followed by oxytocin application recapitulates the pain-related behaviors in the mouse model of uterine pain induced by 3 days of estrogen treatment. (A) Schematic diagram illustrates the protocol for acute intraperitoneal injection of 2-OHE1 and 4-OHE1 to elicit uterine pain in ovariectomized female mice implanted with estrogen pellets. (B-E) Quantification of writhing responses (B), total moving distance (C), total time spent on moving (D) and total time spent on stationary (E) after intraperitoneal injection of 3 nmol 2-OHE1 for 30 min followed by oxytocin application in wt, Trpa1−/− and Trpv1−/− mice. (F-I) Quantification writhing responses (F), total moving distance (G), total time spent on moving (H) and total time spent on stationary (I) after intraperitoneal
injection of 3 nmol 4-OHE1 for 30 min followed by oxytocin application in wt, Trpa1−/− and
Trpv1−/− mice. **p<0.01, ***p<0.001, ****p<0.0001, n=4-6, One-way ANOVA.