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Activation of estrogen receptor α enhances bradykinin signaling in peripheral sensory neurons of female rats.

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2. Running Title Page

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17ß-E_2, 17ß-estradiol; **BK**, bradykinin; **BSA**, bovine serum albumin; **DMSO**, dimethyl sulfoxide; **DPN**, 2,3-bis(4-hydroxyphenyl)propionitrile; **DRG**, dorsal root ganglia; **ER**, estrogen receptor; **GPER**, G protein-coupled estrogen receptor 1; **HBSS**, Hank's balanced salt solution; **i.pl**., intraplantar; **IP**, inositol phosphate; **MPP**, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride; **OVX**, ovariectomized; **PKCε**, protein kinase C epsilon; **PLC**, phospholipase C; **PPT**, 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole; **PWL**, paw withdrawal latency; **siRNA**, small interfering RNA

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Abstract

Numerous studies have demonstrated that females have a higher risk of experiencing several pain disorders with either greater frequency or severity than males. Although the mechanisms that underlie this sex disparity remain unclear, several studies have shown an important role for sex steroids, such as estrogen, in the modulation of nociception. Receptors for estrogen are present in primary afferent neurons in the trigeminal and dorsal root ganglia, and brief exposure to estrogen increases responses to the inflammatory mediator, bradykinin (BK). However, the mechanism for estrogen-mediated enhancement of BK signaling is not fully understood. The aim of the present study was to evaluate the relative contributions of estrogen receptor alpha (ERα), ERβ, and G protein-coupled estrogen receptor 1 (GPER) to the enhanced signaling of the inflammatory mediator, BK, by 17ß-estradiol (17ß-E_2) in primary sensory neurons from female rats in culture (ex vivo) and in behavioral assays of nociception in vivo. The effects of 17ß-E_2 on BK responses were mimicked by ERα selective agonists, blocked by ERα selective antagonists and by siRNA knockdown of ERα. The data indicate that ERα is required for 17ß-E_2-mediated enhancement of BK signaling in peripheral sensory neurons in female rats.

Introduction

Many epidemiological studies have shown that women are at increased risk for several clinical pain disorders and are often more sensitive to experimentally-induced pain than men (for reviews see Craft, 2007; Fillingim et al., 2009). Among the many possible reasons for the disparity in pain responsiveness between men and women, which include cognitive and sociocultural differences, several studies have shown the importance of the sex hormone, estrogen, in regulating nociception (Craft, 2007; Fillingim et al., 2009; Gintzler and Liu, 2012).

Estrogen effects on nociception are complex and often contradictory. In studies of experimental pain in humans, increases, decreases and no change in pain responsiveness during the menstrual cycle or during hormone replacement therapy have been reported (Fillingim et al., 2009). The complexity in these human studies may reflect differences in the way in which the phases of the menstrual cycle were defined, differences in pain models (electrical, thermal, mechanical, etc.), age of the women studied (pre- vs. post-menopausal) and the area of the body tested. However, even in animal studies, there are many contradictory reports on the effects of estrogen on nociception (see Craft, 2007; Gintzler and Liu, 2012 for reviews). For example, when estrogen is administered to ovariectomized (OVX) rats, nocifensive behavior in response to formalin injection into the rat hindpaw is reduced (Hunter et al., 2011). However, When administered to the intrathecal space of intact or OVX female rats, estrogen produced mechanical allodynia and thermal hyperalgesia (Zhang et al., 2012). The nociceptive behavioral response to intraarticular injection of Freund's complete adjuvant to the temperomandibular joint can be enhanced (Kramer and Bellinger, 2009) or reduced (Kou et al., 2011) by estrogen. It is likely that such complex and contradictory actions of estrogen on pain responsiveness reflect differential actions of estrogen on different receptor subtypes and cell populations in the pain

transmission/perception pathways within the central and peripheral nervous systems, along with time-dependent effects (including genomic versus non-genomic signaling) and differences in the type of pain studied.

Of the many possible targets for estrogen within the pain neurotransmission system, studies have suggested that estrogen can alter the function of the primary sensory neurons that respond to noxious stimuli (nociceptors). Receptors for estrogen are expressed by primary sensory neurons (Papka et al., 2001; Bereiter et al., 2005; Chaban and Micevych, 2005; Dun et al., 2009; Liverman et al., 2009b) and treatment of dorsal root ganglia (DRG) neurons in culture with estrogen enhances capsaicin-induced currents (Chen et al., 2004), reduces translocation of protein kinase C subtype epsilon (PKCε) (Hucho et al., 2006) and attenuates ATP-induced calcium currents (Chaban and Micevych, 2005). We have shown that estrogen treatment of sensory neurons in culture from the adult rat trigeminal ganglion enhances signaling by the inflammatory mediator, bradykinin (BK) (Rowan et al., 2010). Interestingly, each of these estrogen effects in sensory neurons occurs rapidly, within minutes, suggestive of rapid onset, non-genomic mechanism mediated by membrane-associated estrogen receptors.

The two primary receptors for estrogen, termed estrogen receptor (ER) α and ERβ, share greater than 60% sequence homology and are best known for their roles as nuclear receptors that regulate gene transcription and protein synthesis (Gibson and Saunders, 2012). ERα and ERβ are differentially expressed throughout the central and peripheral nervous systems (Perez et al., 2003) and are differentially activated by ligands in a tissue-specific manner, a characteristic exploited therapeutically by the selective estrogen receptor modulators (Hall et al., 2001; Nilsson and Koehler, 2005; Nelson et al., 2013). In addition to regulating gene transcription, both ERα

and ERβ are found associated with the plasma membrane where they can rapidly regulate neuronal excitability (Woolley, 2007; Roepke et al., 2011; Srivastava et al., 2011) and can mediate rapid onset, non-genomic signaling to many second messenger systems involved in nociceptive transmission, such as cAMP, calcium and various kinases (Hammes and Levin, 2007; Levin, 2009). In addition, an estrogen-sensitive, G-protein coupled receptor, GPR30 or GPER, recently has been identified that is also capable of rapid onset, non-genomic signaling (Barton, 2012).

We recently have found that 17ß-estradiol (17ß-E_2), rapidly (within minutes) enhances signaling by the inflammatory mediator, BK, in primary sensory neurons in culture and in an animal model of nociception (Rowan et al., 2010). The effect of 17ß-E_2 was mimicked by a membrane impermeable estrogen (17ß-E_2 conjugated with BSA) and not blocked by the protein translation inhibitor, anisomycin, indicating mediation by a membrane-associated ER. In the present work we evaluated the relative contributions of ERα, ERβ, and GPER to the enhanced signaling of the inflammatory mediator, BK, by 17ß-E_2 in adult female rats.

Materials and Methods

Materials

Fetal bovine serum was purchased from Gemini Bioproducts (Calabasas, CA). All other tissue culture reagents were from Invitrogen (Carlsbad, CA). (±)-1-[(3a*R**,4*S**,9b*S**)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3*H*-cyclopenta[*c*]quinolin-8-yl]-ethanone (G-1) and (3a*S**,4*R**,9b*R**)-4-(6-Bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-3*H*-cyclopenta[*c*]quinoline (G-15) were purchased from Tocris Bioscience (Ellisville, MO). 17β-estradiol (17ß-E_2), 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT), 2,3-bis(4-hydroxyphenyl)propionitrile (DPN), 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinylethoxy)phenol]-1H-pyrazole dihydrochloride (MPP), 4-(cyclohexylidenemethylene)-bis-phenol-1,1'-diacetate (cyclofenil), 17β-estradiol-6-(O-carboxymethyl)oxime:BSA (E2-BSA) and all other drugs and chemicals were purchased from Sigma Aldrich (St. Louis, MO).

Animals

The animal study protocol was approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio and conformed to International Association for the Study of Pain and federal guidelines. Ovariectomized (OVX), adult female Sprague-Dawley rats, 200 to 250 g, were purchased from Charles River (Wilmington, MA). Experiments with OVX rats were performed at least two weeks following surgery. Animals were housed with food and water available *ad libitum* before experiments.

Rat trigeminal ganglion culture

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Primary cultures of rat primary sensory neurons were derived from adult OVX female rat trigeminal ganglion and prepared as described previously (Patwardhan et al., 2006; Berg et al., 2007a; Berg et al., 2007b; Rowan et al., 2010). Briefly, rats were sacrificed by decapitation and ganglia were rapidly removed and chilled in Hank's balanced salt solution (HBSS; Ca^{2+}, Mg^{2+} free) on ice. Ganglia were washed with HBSS, digested with 3 mg/ml collagenase for 30 min at 37°C, and centrifuged. The pellet was further digested with 0.1% trypsin for 15 min at 37°C, pelleted by centrifugation (5000g for 5 min), and resuspended in Dulbecco's modified Eagle's medium (high glucose) containing 100 ng/ml nerve growth factor (Harlan, Indianapolis, IN), 10% fetal bovine serum, 1x pen/strep, 1x l-glutamine, and the mitotic inhibitors 7.5 µg/ml uridine and 17.5 mg/ml 5-fluoro-2'-deoxyuridine. After trituration to disrupt tissue clumps, the cell suspension was seeded on polylysine-coated 6 (mRNA), 24 or 48 (IP accumulation) well plates. Media was changed 24 and 48 h after plating, and every 48 h thereafter. Nerve growth factor and serum were removed 24 h prior to experiments. Cells were used on the 5^{th} or 6^{th} day of culture.

Measurement of inositol phosphate accumulation

BK-stimulated inositol phosphate (IP) accumulation in primary sensory neuronal cultures was measured as described previously (Patwardhan et al., 2006; Berg et al., 2007a; Rowan et al., 2010). Cells grown in 24- or 48-well plates were labeled with 2 µCi/ml [^{3}H]myoinositol for 24 h before experiments. After labeling, cells were rinsed three times with 1 ml of HBSS that contained 20 mM HEPES and were pre-incubated in HBSS containing 20 mM HEPES and 20 mM LiCl for 15 min at 37°C in room air. Where indicated, antagonists were added 15 min before agonists, which were added 15 min before BK stimulation (1 nM, 25 min, 37°C). Final volume

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was 250 µl (48 well) or 500 µl (24 well). The incubation was terminated by addition of 500 µl (48 well) or 1 ml (24 well) of ice-cold formic acid, and total [^{3}H]IPs were separated with ion-exchange chromatography and measured with liquid scintillation spectrometry. Data are expressed as accumulation of total IPs (disintegrations per minute) or as a percentage of basal IP accumulation.

siRNA

On-Targetplus SmartPool siRNA for ERα and ERβ, siGLO transfection indicator and DharmaFect 3 transfection reagent were purchased from Dharmacon (Lafayette, CO). siRNA sequences for rat ERα were: GAAUCAAGGUAAAUGUGUA, UCAAGUCGAUUCCGCAUGA, AACCAAUGCACCAUCGAUA, and GCACAAGCGUCAGAGAGAU. siRNA sequences for rat ERβ were: UCGCAAGUGUUAUGAAGUA, GUAAACAGAGAGACACUGA, AAUCAUCGCUCCUCUAUGC, and GCACAAGGAGUAUCUCUGU. 48 h prior to experiments, cultures were transfected with 50 nM siRNA using 1:200 DharmaFECT. 24 h after transfection, media was removed and replaced with serum free media as in other experiments.

Behavioral testing

Paw withdrawal latency (PWL) to a radiant thermal stimulus was measured with a plantar test apparatus (Hargreaves et al., 1988; Rowan et al., 2009; Rowan et al., 2010) by observers blinded to the treatment allocation. Briefly, rats were placed in plastic boxes with a glass floor maintained at 30°C. After a 30 min habituation period, the plantar surface of the hindpaw was exposed to a beam of radiant heat through the glass floor. The rate of increase in temperature was

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adjusted so that baseline PWL values were 10 ± 2 sec; cut-off time was 25 sec. PWL measurements were taken in duplicate (separated by 30 sec) at 5 min intervals. The average of the duplicate measurements was used for statistical analysis. 17ß-E_2 stock solution was diluted in peanut oil. Stock solutions of all other drugs were diluted in saline with or without 2% Tween-20 as indicated. All drugs were administered via intraplantar (i.pl.) injection at a final volume of 50 µl. Where indicated, antagonists were administered 15 min before agonists, which were given 15 min before BK injection. Doses of agonists and antagonists were chosen to produce maximal receptor occupancy based upon maximal concentrations (100 x K_i) reported in the literature (Stauffer et al., 2000; Meyers et al., 2001; Sun et al., 2002; Muthyala et al., 2003; Bologa et al., 2006; Zhao and Brinton, 2007; Dennis et al., 2009) and resulting concentrations following i.pl. injection into the paw assuming a volume of distribution of 1 ml (Rowan et al., 2010).

Data analysis

For cell culture experiments, concentration-response data were fit to a logistic equation (Equation #1) using non-linear regression analysis to provide estimates of maximal response (R_{max}), potency (EC_{50}) and slope factor (n).

$$R = \frac{R_{\max}}{1 + \left(EC_{50} / [A] \right)^{n}}$$

Equation #1

where R is the measured response at a given agonist concentration (A), R_{max} = maximal response, EC_{50} = the concentration of agonist that produces half-maximal response, and n = slope index. Statistical differences in concentration-response curve parameters between groups were analyzed with Student's paired t-test. For behavioral experiments, When only a single concentration was used, statistical significance was assessed using one-way analysis of variance

followed by Dunnet's post-hoc or Student t-test (paired) using Prism software (Graphpad Software, Inc., San Diego, CA). p values less than 0.05 was considered statistically significant.

For behavioral experiments, time-course data were analyzed with two-way analysis of variance, followed by Bonferroni's post-hoc test. Data are presented as mean ± SEM and p values less than 0.05 were considered statistically significant.

Results

17ß-E_2 dose-dependently enhanced BK responses.

Intraplantar injection of 17ß-E_2 to OVX rats at doses up to 1 μg had no effect on basal PWL but dose-dependently enhanced the response of a sub-threshold dose of BK (1 μg, Figures 1A and 1B). As we have shown before (Rowan et al., 2010), BK-induced thermal allodynia was rapid and transient, with peak responses occurring 5 min after BK injection. Peak responses were analyzed to yield an ED_{50} for 17ß-E_2 of 8.85 ng (Figure 1B). In sensory neuronal cultures, a threshold concentration of BK (1 nM) increased IP accumulation by approximately 15% (Figure 2). Although 17ß-E_2 by itself, at concentrations up to 100 nM, did not alter basal IP accumulation, pre-treatment (15 min) of cells with 17ß-E_2 produced a concentration-dependent enhancement of BK-stimulated IP accumulation (Figure 2, EC_{50}= 4.07 nM; E_{max} = 60% above basal).

ERα agonists mimicked the effect of 17ß-E_2 to enhance BK responses.

OVX rats were injected (i.pl.) with the ER subtype-selective agonists, PPT (ERα, Stauffer et al., 2000; Sun et al., 2002), DPN (ERβ, Meyers et al., 2001; Muthyala et al., 2003; Zhao and Brinton, 2007), G-1 (GPER, Bologa et al., 2006), or vehicle 15 min prior to injection with a sub-threshold dose of BK (1 μg, i.pl.). None of the agonists alone affected basal PWL, however, PPT, but not DPN or G1, significantly enhanced the allodynic response to BK (Figure 3). Similarly, 17ß-E_2, PPT, DPN, or G-1 did not alter basal PLC responses in sensory neuron cultures from OVX rats (not shown). However, 15 min pretreatment with 17ß-E_2 or PPT

significantly enhanced BK (1 μM)-stimulated IP accumulation by 2-fold vs. 4.5-fold, respectively (Figure 4).

ERα antagonists blocked the effects of 17ß-E_2, PPT, and 17ß-E_2-BSA to enhance BK responses.

The non-selective ER antagonist, ICI 182780 (1 μg, i.pl.), did not affect basal PWL in OVX rats, but when injected 15 min prior to 17ß-E_2 (100 ng), PPT (2 ng), or 17ß-E_2-BSA (30 μg), ICI 182780 completely blocked the enhancement of BK-induced (1 μg) thermal allodynia (Figure 5). Similarly, the ERα selective antagonist, MPP (1 μg) also blocked the 17ß-E_2- and 17ß-E_2-BSA-mediated enhancement of BK-induced thermal allodynia, without altering PWL on its own (Figure 6). By contrast, the 17ß-E_2-mediated enhancement of BK-induced thermal allodynia was unaffected by the ERß antagonist, cyclofenil, or the GPER antagonist, G-15, at doses chosen to produce maximal receptor occupancy (100 x Ki).

When tested in sensory neuron cultures from OVX female rats at concentrations (100 x Ki) chosen to produce maximal receptor occupancy (Stauffer et al., 2000; Meyers et al., 2001; Sun et al., 2002; Muthyala et al., 2003; Bologa et al., 2006; Zhao and Brinton, 2007; Dennis et al., 2009), only ICI 182780 (ERα and ERß, 30 nM) and MPP (ERα, 300 nM) blocked 17ß-E_2 (50 nM)-mediated enhancement of BK (1 nM)-stimulated IP accumulation (Figure 7). Cyclofenil (ERβ, 10 nM) and G-15 (GPER, 2 μM) were without effect. None of the antagonists alone altered basal IP accumulation.

Expression of ERα is required for 17ß-E_2-mediated enhancement of BK signaling.

Sensory neuron cultures were treated for 48 h with siRNA (50 nM) selective for ERα, ERβ, or non-targeting control siRNA (siGLO). RT-PCR analysis of total mRNA showed that treatment with siRNA for ERα or ERß reduced selectively their respective mRNA by 47% and 38%, respectively (Figure 8A). Treatment with ERα or ERß siRNA did not alter threshold BK (1 nM)-stimulated IP accumulation (BK stimulation, percent above basal: siGLO, 25 ± 4; ERα siRNA, 31 ± 5; ERβ siRNA, 30 ± 4). In cells transfected with control siRNA, 17ß-E_2 enhanced BK-stimulated IP accumulation as shown before. Consistent with experiments using subtype-selective agonists and antagonists, the 17ß-E_2-mediated enhancement of BK-stimulated IP accumulation was blocked by siRNA directed toward ERα but not ERβ (Figure 8B).

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Discussion

Although ERα and ERβ share a significant amount of sequence homology, especially in the DNA-binding domain, their differential tissue expression and differential sensitivity to ligands creates an exciting area of potential therapeutic intervention for a variety of disease states. Although both ERα and ERß have been strongly implicated in regulating nociception (Craft, 2007; Coulombe et al., 2011; Gintzler and Liu, 2012), such regulation of pain responsiveness by estrogen is complex and often contradictory. Such complexity is likely due to differential effects mediated by ERα and ERß that are expressed at multiple levels of the pain neurotransmission system (Coulombe et al., 2011). Additionally, effects of estrogen may differ depending upon the type of pain studied (Craft, 2007). Thus, to begin to clarify estrogen's role in pain, it is important to study estrogen's effects in defined regions of the pain processing system.

Nociceptors respond to intense, potentially tissue damaging stimuli and send signals to the CNS that are interpreted as pain and thus are generally the first stage in the pain-processing system. Nociceptors express three receptor subtypes that respond to estrogen, ERα, ERß and GPER (Papka et al., 2001; Bereiter et al., 2005; Chaban and Micevych, 2005; Dun et al., 2009; Liverman et al., 2009b) and therefore estrogen is in a position to regulate pain neurotransmission at this initial stage. We recently found that 17ß-E_2 rapidly (within 15 min) enhances the responsiveness of nociceptors in vitro and in vivo to activation by BK (Rowan et al., 2010). The rapid-onset effect of 17ß-E_2 is mediated by a non-genomic mechanism via a membrane-associated receptor (Rowan et al., 2010). Here we provide evidence that the rapid-onset effect of 17ß-E_2 to enhance nociceptor sensitivity to BK is mediated by the ERα receptor subtype.

17ß-E_2 dose-dependently enhanced BK-induced thermal allodynia in the rat hindpaw in vivo and PLC activity in sensory neurons in culture, consistent with our previous report (Rowan et al.). Only the ERα-selective agonist, PPT, mimicked the enhancement of BK responses seen with 17ß-E_2. The effects of 17ß-E_2, PPT, and the membrane impermeable 17ß-E_2-BSA were blocked by the ERα-selective antagonist, MPP, but not by the ERβ-selective antagonist, cyclofenil, or the GPER antagonist, G-15. Neither the ERβ-selective agonist, DPN, nor the GPER-selective agonist, G-1, enhanced BK-stimulated thermal allodynia when injected into the hindpaw at doses chosen to produce maximal receptor occupancy. Collectively, these data suggest that the estrogen receptor that mediates rapid-onset enhancement of BK responses by estrogen in peripheral sensory neurons is ERα.

The utility of selective agonists and antagonists to identify receptors that mediate a response is limited by the degree of ligand selectivity for the target receptor and the dose/concentration of the ligands used. Although the affinities of the drugs used in the present study provide the highest available level of selectivity for their respective targets, there is some concern because affinity/selectivity of ER ligands has generally been characterized with the nuclear ERs that regulate gene transcription responses. The affinity of ligands for a receptor can be influenced by the molecular partners of the receptor. For example, the influence of a G protein on the binding affinity of agonists for 7 transmembrane-spanning receptors is well known. Thus, it is possible that the selectivity of ligands for ERs may differ depending upon the location (membrane versus nuclear) and molecular partners of the ERs. To address this issue and to support the pharmacological results, we used SmartPool siRNA to selectively reduce ERα or ERβ expression in sensory neurons in culture. Treatment with siRNA selectively reduced expression of ERα or ERβ mRNA. Whereas non-targeting, control siRNA, or treatment with

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siRNA directed at ERβ had no effect on either the baseline BK-stimulated IP accumulation or the 17ß-E_2-mediated enhancement of BK responses, treatment with siRNA directed at ERα completely abolished the enhancing effect of 17ß-E_2 on BK-stimulated PLC activity, without affecting the baseline BK response. These results support the conclusion that the ERα subtype mediates the enhancing effect of estrogen on BK responses in peripheral sensory neurons.

In addition to ERα, ERß and GPER are expressed in sensory neurons and both DPN and G-1 produced small enhancements of BK-stimulated PLC activity when applied to sensory neurons in culture. The lack of effect of DPN and G-1 to enhance BK-stimulated thermal allodynia in vivo suggests that the magnitude of the effect on BK signaling may be below threshold needed to produce behavioral effects on thermal allodynia. Alternatively, ERß and GPER could be expressed by sensory neurons that are not responsive to thermal stimulation but could regulate responsiveness to noxious mechanical stimulation. Also, differences in the responses to DPN and/or G-1 between the experiments with sensory neurons of the trigeminal ganglion and the behavioral assay could reflect differences between the trigeminal versus the dorsal root ganglia sensory neurons, which mediate the paw withdrawal response to drug administration in the hindpaw.

The mechanism by which activation of membrane-associated ERα rapidly enhances BK signaling in peripheral sensory neurons is not known. A large number of signaling pathways have been shown to be regulated by membrane-associated ERs in a variety of tissues, including mitogen-activated protein kinases, phosphatidylinositol 3-kinase, endothelial nitric oxide synthase activation, cAMP production and intracellular calcium mobilization (Hammes and Levin, 2007; Levin, 2009). However, little is known about the contribution of these signaling

pathways in mediating rapid effects of estrogen on nociception. In one study, rapid enhancement of inflammation-induced mechanical allodynia of the masseter muscle by estrogen was shown to be mediated by activation of extracellular signal-regulated kinase (ERK) in sensory neurons (Liverman et al., 2009a). Additional experiments are needed to delineate the signaling pathway(s) regulated by membrane-associated ERα activation that mediate effects of estrogen to enhance BK-stimulated signaling in peripheral sensory neurons.

Many studies have now shown that women experience a disproportionate amount of pain and that estrogen may be a contributory factor. Estrogen has been implicated in a variety of pain-causing conditions, including arthritis, migraine, and temporomandibular disorder. However, the effect of estrogen on pain processing is multifaceted and complex, with reports of both pro- and anti-nociceptive actions. Adding to the complexity are the multiple ER subtypes, genomic (long-term) and non-genomic (rapid onset), and developmental and activational effects of estrogen. Consequently, unraveling the complexity of estrogen's role in pain, as with many other estrogen-regulated physiological processes, requires careful dissection of effects at multiple levels of the pain processing system. Our results indicate that acute activation of membrane-associated ERα in peripheral sensory neurons by estrogen rapidly enhances thermal allodynia elicited by the inflammatory mediator, BK. It is possible that targeting membrane-associated ERα with selective antagonists could be effective analgesics for inflammatory pain in women.

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Authorship Contributions:

Participated in research design: Berg, Clarke, Hargreaves, Roberts, and Rowan

Conducted experiments: Berg and Rowan

Performed data analysis: Berg, Clarke, and Rowan

Wrote or contributed to the writing of the manuscript: Berg, Clarke, Hargreaves, Roberts, and Rowan

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Footnotes:

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Figure Legends

Figure 1

Local application of 17β-estradiol (17ß-E_2) rapidly and dose-dependently enhances bradykinin-induced thermal allodynia in OVX rats. **A**, OVX rats received intraplantar injections (50 µl) of 17ß-E_2 (doses indicated) or vehicle 15 min before injection with a subthreshold dose of bradykinin (BK, 1 µg). Paw withdrawal latency (PWL) in response to a radiant heat stimulus applied to the ventral surface of the hindpaw was measured in duplicate at 5 min intervals before (baseline) and following each injection. Data are expressed as the change (sec) from individual pre-injection baselines (10 ± 2 sec) and represent the mean ± SEM of 4-6 animals per group. **,***; $p<0.01$, 0.001 vs. vehicle by two-way ANOVA with Bonferroni post-hoc analysis. **B**, dose-response curve for 17ß-E_2 enhancement of BK-induced thermal allodynia. Data points are from **A** at the time of peak BK response (5 min post-injection) as a function of 17ß-E_2 dose. ED_{50} = 8.85 ng.

Figure 2

Local application of 17β-estradiol rapidly and dose-dependently enhances BK-stimulated PLC activity in cultures of peripheral sensory neurons from female rats. Peripheral sensory neuron cultures, prepared from OVX rats, were treated with vehicle (0.1% DMSO) or 17ß-E_2 (concentrations indicated) for 15 min (37ºC) before addition of BK (1 nM) or vehicle (HBSS). Total IP accumulation after 25 min was measured as described under *Materials and Methods*.

Data are expressed as the total counts (dpm) and represent the mean ± SEM of three experiments.

Figure 3

BK-induced thermal allodynia is enhanced by the ERα-selective agonist, PPT, but not by the ERβ-selective agonist, DPN, or the GPER-selective agonist, G1, in OVX rats. Separate groups of OVX animals received intraplantar injections of PPT (2 or 20 ng), DPN (4 or 40 ng), G1 (0.1 or 1 μg), or vehicle (0.1% DMSO, 2% Tween-20) 15 min before injection with a subthreshold dose of BK (1 μg). PWL was measured in duplicate at 5 min intervals before (baseline) and after each injection. Data are expressed as the change (sec) from individual pre-injection baseline (10 ± 2 sec) and represent the mean ± SEM of 4-6 animals per group. ***, $p<0.001$ vs. Veh by two-way ANOVA with Bonferroni post-hoc analysis.

Figure 4

Activation of ERα enhances BK-stimulated PLC activity in female peripheral sensory neuron cultures. Peripheral sensory neuron cultures from OVX rats were treated with vehicle (0.1% DMSO), 17ß-E_2, PPT, DPN, or G1 (concentrations indicated) for 15 min (37ºC) before addition of BK (1 nM) and further incubation for 25 min (37ºC). Total IP accumulation was determined as described under *Materials and Methods*. Data are expressed as percentage of the

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BK response and represent the mean ± SEM of 6-8 experiments. Basal IP accumulation = 310 ± 19 dpm; BK-stimulated IP accumulation = 408 ± 25 dpm (31% over basal).

Figure 5

17β-estradiol, PPT, and 17ß-E_2-BSA enhancement of BK-induced thermal allodynia is blocked by the ER antagonist, ICI182780. Separate groups of OVX animals received intraplantar injections of the non-selective ER antagonist, ICI182780 (ICI, 1 μg), or vehicle (0.1% EtOH) 15 min prior to injection with 17ß-E_2 (100 ng), PPT (2 ng), or 17ß-E_2-BSA (30 μg). 15 min later, animals received intraplantar injections of BK (1 μg). PWL was measured in duplicate at 5 min intervals before (baseline) after each injection. Data are expressed as change from individual preinjection baselines and represent the mean ± SEM of 4-6 animals per group. ***, $p<0.001$ respective Veh vs. ICI by two-way ANOVA with Bonferroni post-hoc analysis.

Figure 6

17ß-E_2 and 17ß-E_2-BSA-mediated enhancement of BK-induced thermal allodynia is blocked by the ERα antagonist, MPP, but not by antagonists of ERß, cyclofenil, or GPER, G15. Separate groups of OVX animals received intraplantar injections of MPP (1 μg), cyclofenil (1 ng), G15 (1 μg) or vehicle (0.1% EtOH) 15 min prior to injection with 17ß-E_2 (100 ng) or 17ß-E_2-BSA (30 μg). 15 min later, animals were injected with BK (1 μg). PWL was measured in duplicate at 5 min intervals before (baseline) and after each injection. Data are expressed as change (sec) from individual pre-injection baselines and represent the mean ± SEM of 4-6

animals per group. ***, $p<0.001$ vs. all other groups by two-way ANOVA with Bonferroni post-hoc analysis.

Figure 7

17ß-E_2-mediated enhancement of BK-stimulated PLC signaling is blocked by the non-selective antagonist, ICI 182780, and by the ERα-selective antagonist, MPP, but not by the ERβ- or the GPER-selective antagonists, cyclofenil and G15, respectively. Cultures of peripheral sensory neurons from OVX rats were treated with vehicle (0.1% DMSO) or antagonist (ICI 182780, 30 nM; MPP, 300 nM; cyclofenil, 10 nM; G15, 2 μM) 15 min before treatment with vehicle (0.1% DMSO) or 17ß-E_2 (50 nM). 15 min after 17ß-E_2 addition, cells were treated with BK (1 nM) for 25 min and total IP accumulation was determined as described under *Materials and Methods*. Data are expressed as percent of BK stimulation and represent the mean ± SEM of 5-6 experiments. *, $p< 0.05$ by one-way ANOVA with Bonferroni post-hoc analysis. Basal dpm 470 ± 23.1, BK dpm 578 ± 34.4 (23% stimulation over basal).

Figure 8

ERα is required for 17ß-E_2-mediated enhancement of BK- stimulated PLC activity in female peripheral sensory neuron cultures. Peripheral sensory neuron cultures from OVX rats were incubated with 50 nM siRNA or siGLO control for 48 hr prior to experiments as described in *Materials and Methods*. Total mRNA was isolated, amplified by RT-PCR and separated by agarose gel electrophoresis as described *Materials and Methods*. **A)** Representative agarose gel

bands acquired using primers specific to ERα (top row), ERß (middle row) or ß-actin (bottom row) following treatment with siGLO (left two columns), ERα siRNA (middle two columns) or ERß siRNA (right two columns). **B)** Quantification of band densities normalized to β-actin. Data represent the mean ± SEM of 3 experiments. **, ***; $p<0.01$, 0.001 vs. Veh by one-way ANOVA with Bonferroni. **C)** Following siRNA treatment, peripheral sensory neurons cultures were treated with vehicle (0.1% DMSO) or 17ß-E_2 (50 nM) for 15 min (37ºC) before addition of BK (1 nM) and further incubation for 25 min. Total IP accumulation was determined as described under *Materials and Methods*. Data are expressed as percent of BK stimulation and represent the mean ± SEM of 8 experiments. *, $p< 0.05$ by one-way ANOVA with Bonferroni post-hoc analysis.

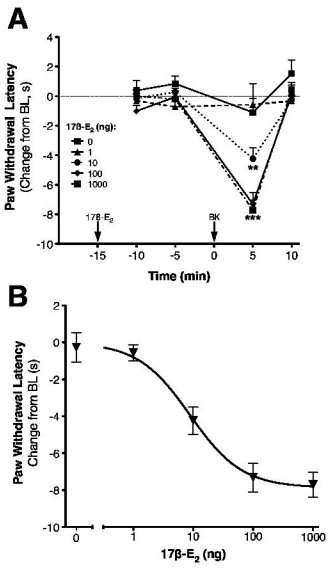

A
Paw Withdrawal Latency
(Change from BL, s)
17β-E2 (ng):
0
1
10
100
1000
17β-E2
BK
**
***
Time (min)
B
Paw Withdrawal Latency
Change from BL (s)
17β-E2 (ng)

Figure 1

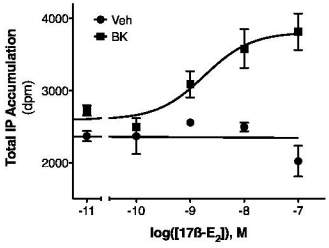

Veh
BK
Total IP Accumulation (dpm)
4000
3000
2000
-11
-10
-9
-8
-7
log([17β-E2]), M

Figure 2

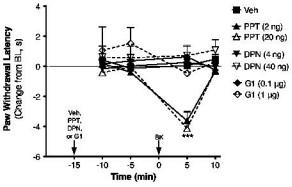

Paw Withdrawal Latency
(Change from BL, s)
4
2
0
-2
-4
-6
Veh, PPT, DPN, or G1
BK
***
-15
-10
-5
0
5
10
Time (min)
Veh
PPT (2 ng)
PPT (20 ng)
DPN (4 ng)
DPN (40 ng)
G1 (0.1 µg)
G1 (1 µg)

Figure 3

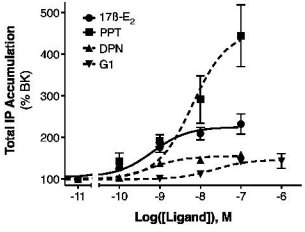

17ß-E2
PPT
DPN
G1
Total IP Accumulation
(% BK)
500
400
300
200
100
-11
-10
-9
-8
-7
-6
Log([Ligand]), M

Figure 4

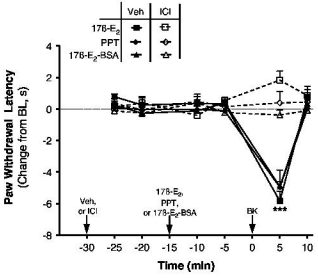

Veh
ICI
17β-E2
PPT
17β-E2-BSA
Paw Withdrawal Latency
(Change from BL, s)
4
2
0
-2
-4
-6
-8
Veh,
or ICI
17β-E2,
PPT,
or 17β-E2-BSA
BK
***
-30
-25
-20
-15
-10
-5
0
5
10
Time (min)

Figure 5

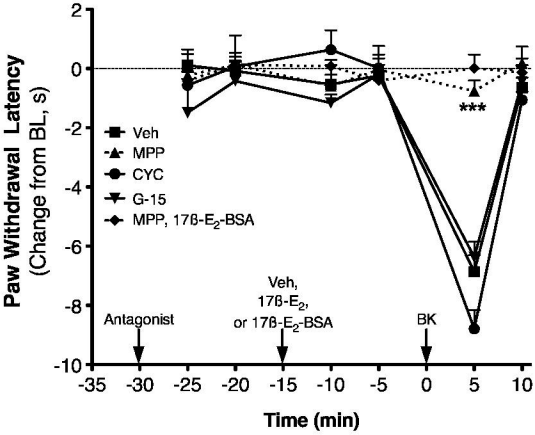

Paw Withdrawal Latency
(Change from BL, s)
2
0
-2
-4
-6
-8
-10
Veh
MPP
CYC
G-15
MPP, 17ß-E2-BSA
***
Antagonist
Veh,
17ß-E2,
or 17ß-E2-BSA
BK
-35
-30
-25
-20
-15
-10
-5
0
5
10
Time (min)

Figure 6

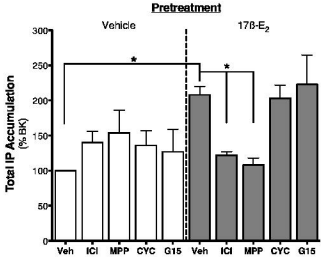
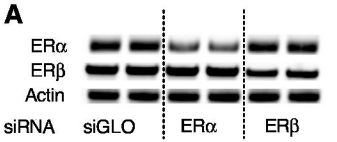
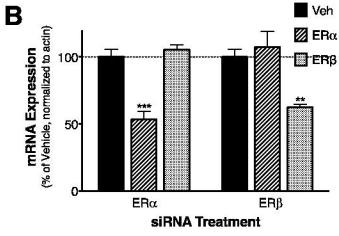

Pretreatment
Vehicle
17β-E2
Total IP Accumulation
(% BK)
300
250
200
150
100
50
0
*
*
Veh
ICI
MPP
CYC
G15
Veh
ICI
MPP
CYC
G15

Figure 7


A
ERα
ERβ
Actin
siRNA
siGLO
ERα
ERβ


B
Veh
ERα
ERβ
mRNA Expression
(% of Vehicle, normalized to actin)
100
50
0
***
**
ERα
ERβ
siRNA Treatment

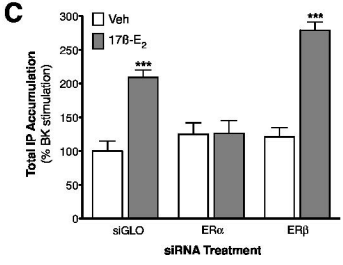

C
Veh
17β-E2
Total IP Accumulation
(% BK stimulation)
300
250
200
150
100
50
0
***
***
siGLO
ERα
ERβ
siRNA Treatment

Figure 8