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Activation of membrane estrogen receptors attenuates opioid receptor-like1 receptor-mediated antinociception via an ERK-dependent non-genomic mechanism

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Abstract

To our knowledge, the present data are the first to demonstrate that activation of membrane estrogen receptors (mERs) abolishes opioid receptor-like 1 (ORL1) receptor - mediated analgesia *via* extracellular signal-regulated kinase (ERK)-dependent non-genomic mechanisms. Estrogen was shown previously to both attenuate ORL1- mediated antinociception and down-regulate the ORL1 gene expression. The present study investigated whether non-genomic mechanisms contribute to estrogen-induced attenuation of ORL1-mediated antinociception by the mERs GPR30, Gq-coupled mER, ERα, and ERβ. E2-BSA (0.5 mM), a membrane impermeant analogue of estradiol, injected intrathecally immediately prior to Orphanin FQ (OFQ;10 nmol), the endogenous ligand for the ORL1 receptor, abolished OFQ's antinociceptive effect in both male and ovariectomized (OVX) female rats, assessed using the heat-induced tail-flick assay. This effect was not altered by protein synthesis inhibitor, anisomycin (125 μg), given intrathecally 15 minutes prior to E2BSA and OFQ. Intrathecal application of selective receptor agonists permitted the relative contributions of various estrogen receptors in mediating this blockade of the antinociceptive response of OFQ. Activation of GPR30, Gq-mER, ERα, but not ERβ abolished ORL1-mediated antinociception in males and OVX females. E2BSA produced a parallel and significant increase in phosphorylation of ERK 2 only in OVX females, and pre-treatment with MEK/ERK 1/2 inhibitor, U0126 (10μg), blocked the mER-mediated abolition of ORL1-mediated antinociception in OVX females. Taken together, the data are consistent with the interpretations that mER activation attenuates ORL1-mediated antinociception through a non-genomic, ERK 2 - dependent mechanism in females.

Introduction

Numerous studies have reported sex-related differences in pain syndromes, analgesic efficacy, pain perception, and pain control (Gear et al. 1996; Berkley, 1997; Fillingim and Gear, 2004; Hucho et al., 2006; Greenspan et al., 2007; Mogil, 2012). Our laboratory (Claiborne et al., 2006; Thompson et al., 2008) and others (Fillingim and Ness, 2000;

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Conflicts of Interest
The authors have no conflicts of interest to declare.

LeResche et al., 2003; Ji et al., 2008; Liverman et al., 2009) have shown that sex hormones play a critical role in pain perception and control. Estrogen has been shown to differentially modulate antinociceptive effects of G protein coupled receptors (GPCRs) such as opioid (Claiborne et al., 2006; Peckham and Traynor, 2006; Craft et al., 2008; Loyd et al., 2008; Lawson et al., 2010; Liu et al., 2011) and α_2 – noradrenergic receptors (Mitrovic et al., 2003; Nag and Mokha, 2006; Thompson et al., 2008).

The present investigation focuses on the opioid receptor-like 1 (ORL1) receptor, which is expressed in the dorsal horn (Bunzow et al., 1994; Mollereau et al., 1994) and couples to inhibitory G proteins ($G\alpha_i/\alpha_o$) to decrease voltage-gated calcium channel conductance and facilitate receptor-operated potassium channel conductance. Orphanin FQ (OFQ) (Meunier et al., 1995; Reinscheid et al., 1995), an endogenous ligand for the ORL1 receptor, produces pronociceptive effects when administered supra-spinally (Grisel et al., 1996; Wang et al., 1999) and antinociceptive effects when administered at the level of the spinal cord (Stanfa et al., 1996; Wang et al., 1999; Xu et al., 1996). In addition, we have previously shown that estrogen attenuates OFQ- induced antinociception (Flores et al., 2001; Claiborne et al., 2006) possibly via a genomic mechanism comprising down regulation of the ORL1 gene expression (Flores et al., 2003). However, recent discoveries of membrane estrogen receptors (mERs), namely post-translationally modified classical, cytosolic ERα and ERβ (Pappas et al., 1995; Razandi et al., 1999. Levin, 2009) as well as two membrane bound ERs – GPR30 (Thomas et al., 2005; Filardo et al., 2007; Dun et al., 2009) and Gq-coupled mER (Gq-mER) (Qui et al., 2003) provide a possible molecular strategy for mediating non-genomic effects of estrogen (Raz et al., 2008; Levin, 2009; Roepke et al., 2009). mERs initiate intracellular signaling cascades that uncouple receptors from their effector systems (Kelly et al., 2002) or activate kinases that modulate ion channel activity (Kelly et al., 2003) and are thus capable of affecting ORL1-mediated antinociception.

Hence, we investigated whether activation of mERs contributes to the estrogen-induced attenuation of ORL1-mediated antinociception, as well as mER-induced changes in levels of activated extracellular signal regulated kinase 1/2 (ERK 1/2) and protein kinases A and C (PKA, PKC).

Experimental Procedures

Animals

Adult Sprague-Dawley male and ovariectomized (OVX) female rats (about 250- 274g) were obtained from Harlan Inc. (Indianapolis, IN, USA). They were housed in a temperature controlled room (~22°C) under 12 hour light/dark cycle (lights on at 7:00 AM and lights off at 7:00 PM) in the animal care facility at Meharry Medical College certified by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). Food and water were available *ad libitum*. Experimental protocols were approved by the Institutional Animal Care and Use Committee of Meharry Medical College and followed the established guidelines of the National Institute of Health Guide for the Care and use of Laboratory Animals. All efforts were made to minimize stress to the animals and the number of animals used.

Implantation of intrathecal cannulae

OVX female animals were given a 2 week recovery period prior to surgical implantation of cannulae. As previously described by our laboratory (Claiborne et al., 2006; Thompson et al., 2008; Lawson et al., 2010) and others (Yaksh and Rudy,1976), animals were surgically prepared under ketamine and xylazine anesthesia (72 and 4 mg/kg, i.p., respectively). Their heads were shaved and secured in a stereotaxic frame (David Kopf Instruments, Tujunga,

CA) and an incision was made above the atlantooccipital membrane which was then cleared to expose the dura. A stretched PE-10 cannula (Intramedic, Clay Adams, Parsippany, NJ; dead volume 10 μl) was inserted into the subarachnoid space through a small opening in the dura. The cannula was pushed to a length of 8.5 cm to reach the lumbosacral enlargement and was secured by dental cement. The wound was sealed with suture clips and animals were kept warm on a heating blanket until they regained consciousness. They were allowed to recover for a minimum of 5 days before the nociceptive testing. Animals showing any signs of neurological impairment were euthanized immediately with 150 mg/kg (i.p.) of sodium pentobarbital or Buthenasia (Med-Vet International, Mettawa, IL). The position of the cannula was confirmed at the end of the experiment by administering 10 μl of 2% lidocaine which temporarily paralyzed the hind limbs, and by visual examination of 1% pontamine sky blue dye spread.

Tail Flick Test

Since a large majority of studies in sex-related differences in pain utilize a nociceptive heat-induced tail flick assay (D'Amour and Smith, 1941), we decided to choose the same so our results could be closely relatable to the available literature in the field for direct comparison. The tail flick assay models acute thermal pain and has been successfully utilized for understanding basic pain mechanism as well as drug development. Tail flick latencies (TFL) were measured using an automated analgesia meter (model 33T, IITC Woodland Hills, CA). Prior to testing, animals were loosely restrained in a Plexiglas cylinder and acclimatized to the restraint cylinder for 30 minutes. A radiant heat source was applied to the dorsal surface, 4–7cm from the tip of the rat's tail, at three separate spots to prevent sensitization as a result of heating the same spot in succession. Further, no visible evidence of tissue damage was observed at the end of the experiment. The heat intensity was preset to produce baseline TFLs between 3 and 6 seconds. An automatic cut off latency of 15 seconds was preset to prevent tissue damage. Three baseline tail flick latencies were recorded prior to intrathecal injections at 2 minute intervals, thereafter latencies were recorded for 20 minutes (previously reported antinociceptive response duration of OFQ by Claiborne et al. (2006)). Further, baseline latencies did not change significantly during the testing period as demonstrated previously in vehicle control experiments (Claiborne et al., 2006).

Drugs

Each drug was administered slowly (over a 5 second period) intrathecally with a 20 or 50 μl Hamilton microsyringe by hand in a volume of 10 μl at time "0" unless stated otherwise. OFQ (10 nmol), the endogenous ligand for the ORL1 receptor, dose was selected based on our previously reported dose response curves that produced a robust antinociceptive effect in the tail flick assay (Claiborne et al. 2006).

E2- BSA [β-Estradiol 6-(O-carboxymethyl) oxime: Bovine serum albumin (BSA)], a membrane impermeant analog of estradiol, was administered to target all membrane estrogen receptors. Proestrous (0.36 nM) and diestrous (0.09 nM) equivalent doses of estrogen (in E2BSA) were calculated from previously reported plasma levels of estradiol in normally cycling rats during proestrous (∼100pg/mL)and diestrous stages (∼25 pg/mL) (Butcher et al., 1974). These w/v concentrations convert directly to the molar solutions used in the present study using 272.39 as the formula weight of E2BSA. Molarities used are of E2 in E2BSA. The highest dose of E2BSA (0.5 mM) is in fact the most commonly used dose of estrogen and was derived from previously reported intrathecally applied estrogen that enhanced baroreflex function and autonomic tone and evoke action potentials in spinal neurons (Saleh et al., 2000; Zhang et al., 2012).

G-1 is a selective agonist for the GPR30 receptor; the (0.25 nM) dose was derived based on the binding affinity of G-1 to GPR30 (Bologa et al., 2006). G-15, a GPR30 antagonist, was administered 5 minutes prior to nociceptive testing. G-15 dose (1 μM) was previously shown to block anti-depressive effects of G-1 (Dennis et al., 2009). STX (10 nM), a Gq-mER selective agonist with ∼20X higher affinity than E2, was previously shown to attenuate the γ-aminobutyric acid ($GABA_B$) receptor-mediated response in proopiomelanocortin (POMC) neurons (Qui et al., 2003; Qui et al., 2006). Doses of propylpyrazoletriol (PPT), an ERα-selective ligand; and diarylpropionitrile (DPN), an ERβ-selective ligand, (100 nM) were selected based on previous reports that PPT and DPN facilitated spinal reflex potentiation in the urethra of anesthetized rats (Peng et al., 2009). Anisomycin (125 μg), a protein synthesis inhibitor, was administered 15 minutes prior to drug injection. The dose and time-point employed nearly completely blocks *de novo* protein synthesis (>90%) (Grollman, 1967; Rosenblum et al., 1993; Miletic et al., 2010). Since mER-induced new gene expression was not expected to occur in the given time frame, a positive anisomycin control could not be employed. Such control is usually employed in studies where known genomic changes are expected to occur (García-DeLaTorre et al. 2009; Baumbauer et al. 2006; Rosenblum et al., 1993; Flood et al. 1973). Moreover, intrathecal anisomycin injection protocol employed in the present study has been previously shown to block peripheral nerve injury-induced accumulation of Shank1 protein in the dorsal horn of lumbar spinal cord in the rat (Miletic et al., 2010). U0126 (10 μg), a MEK/ERK 1/2 inhibitor (Wang et al., 2011), and U0124 (10μg), an inactive analog of U0126 (Lai et al., 2011), were administered 30 minutes prior to drug injection. OFQ, G-1, G15, PPT, DPN, U0126, and U0124 were obtained from Tocris (Ellisville, MO) whereas; E2-BSA and anisomycin were obtained from Sigma -Aldrich (St. Louis, MO). Dr. Martin Kelly at Oregon Health Sciences University generously provided STX. Drugs were dissolved in PBS (filtered E2BSA), double distilled boiled water (OFQ), < 1% ethanol (G-15, PPT, DPN), or < 10% DMSO (G1, STX, anisomycin, U0126 and U0124). Appropriate vehicles were used to control for the drug as well as volume effects which were not significantly different from the baseline latencies. The highest volume (40 μl) delivered intrathecally in this study did not affect TFLs for up to 90 min in our pilot experiments conducted with saline injections (data not shown)

Tissue harvesting and preparation

Separate groups of OVX and male animals were given an intrathecal injection of E2BSA +veh (n=3), veh+OFQ (n=3), or E2BSA + OFQ (n=3) and three tail flick readings were recorded to confirm the drug effect. After TFL readings were recorded, animals were deeply anesthetized with sodium pentobarbital (150 mg/kg; i.p.), and the lumbosacral spinal cord was collected in RNA*later* (0.5 ml; Ambion, Austin, TX, USA) and stored at −80 °C for further processing. The above-mentioned procedures were completed by 6 minutes after administration of drugs. Total lumbosacral spinal cord tissue was homogenized for 2 min in RIPA lysis buffer containing phosphatase and protease inhibitor cocktails (Santa Cruz Biotechnology, Inc., Dallas, TX) using an overhead Dounce homogenizer.

Western blot analysis

Total protein content of each sample was determined using a BCA protein assay kit (Biorad, Hercules, CA). The absorbance was read at 650 nm on an Emax Precision Microplate Reader (Molecular Devices, Sunnyvale, CA) and protein concentrations were calculated against a BSA standard curve. Equal amount of protein (60 μg/30 μl) for each sample was subjected to SDS-PAGE (Invitrogen; 4–12% Bis-Tris gel; 200 V for 50 min) after heating at 65 °C for 10 min on a dry heat block. Protein bands were transferred onto PVDF membrane, blocked for 1 h in 5% non-fat dry milk in TBST (Tris buffer saline- 10% Tween-20), and incubated for 48 h at 4°C with the following primary antibodies (Jones and Sorkin, 2005): anti-PKA antibody, NT (1:1000) and anti-phospho-PKA antibody, RII (Ser96) (1:500,

Millipore, Temecula, CA); anti-PKC gamma antibody (1:1000) and anti-phospho PKC gamma (pan) pThr51 (1:500, Pierce, Rockford, IL). Anti-p44/42 MAPK (ERK1/2) (1:1000) and anti-phospho p44/42 MAPK (ERK1/2) (Thr202/Try204) (1:1000, Cell Signaling Technology, Danvers, MA) were incubated for 24 h at 4°C; anti- actin (1:1000, Sigma, St Louis, MO) was incubated overnight at 4°C. After washing, the blots were incubated for 1h at RT with HRP-conjugated bovine anti-rabbit secondary antibody (1:7500, Sigma), and developed using Super Signal West Dura Extended Duration® (Thermo Scientific) and visualized with UVP Epi Chemi II Darkroom (Ultra Violet Products Limited, Upland, CA, USA). Between each immunoblotting, membranes were stripped using Reblot Plus (Millipore, Temecula, CA). Densitometry was performed on the bands using Labworks Image Acquisition and Analysis Software (Bioimaging Systems, Upland, CA, USA). Densities of phosphorylated and total protein kinases were normalized to actin and presented as a ratio of normalized phosphorylated to total protein.

Data analysis

Data were analyzed using SPSS (SPSS Inc., Chicago, IL). All behavior measures were submitted to ANOVA corrected for repeated measures with appropriate between- group (drug) and within- group (time) factors, and dependent variable (TFL). The number of animals in each group was 3–9. Area under the curve (AUC) was calculated by trapezoid method using Prism (Graphpad Software, Inc., San Diego, CA) for time course plots to obtain a single measure of the overall drug response. The data obtained from Western blotting studies and AUC were analyzed by one-way ANOVA. *A post hoc* test (Fisher's LSD) was employed for intergroup comparisons where necessary and only when ANOVA yielded a significant main effect. A *P*-value of <0.05 was considered significant. Data were plotted as mean ± S.E.M.

Results

OVX females and males have comparable basal nociception

To assess whether basal nociception differed between groups, we compared baseline TFLs at times "−6, −4, and −2" in OVX female and male rats. As shown in Fig. 1, 3, 4, and 5, baseline TFLs were comparable for all groups. ANOVA yielded no significant main effects of group ($F_{(41, 168)} = 1.74$; p=0.08), time ($F_{(2, 336)} = 0.54$; p=0.59), or interaction between group x time ($F_{(82, 336)} = 1.09$; p=0.30). These findings provide evidence that the basal nociception as measured in our studies does not intrinsically differ between any groups employed in this study.

Activation of mERs in the spinal cord rapidly and non-genomically attenuates ORL1-mediated spinal antinociception in OVX females and males

In order to assess whether mERs contribute to estrogen-induced attenuation of ORL1-mediated antinociception in the spinal cord, E2BSA (0.5 mM) was injected intrathecally (i.t.) followed immediately by OFQ (10 nmol) in OVX female and male rats and tail flick latencies (TFLs) were recorded (Fig.1A). ANOVA yielded significant main effects of group ($F_{(5, 25)} = 100.32$; p=0.00), time ($F_{(13, 325)} = 15.00$; p=0.00), and interaction between group x time ($F_{(65, 325)} = 8.60$; p=0.00). *Post hoc* comparisons revealed that interaction stemmed from the fact that after three comparable baselines, veh+OFQ treated OVX (n=6) and male (n=7) groups had significantly higher tail flick latencies as compared to the rest of the groups (Figure 1A). The effect of OFQ was seen within 2 min of injection and persisted for the duration (20 min) of the experiment. E2BSA abolished the antinociceptive effect of OFQ in both OVX (n=4) and males (n=5). E2BSA+veh treatment did not alter TFLs in OVX (n=4) or males (n=5), suggesting that E2BSA does not produce pronociceptive effects that would have confounded the interpretation of data. mER activation rapidly abolished ORL1-

mediated antinociception in both OVX female and male groups, suggesting that the mechanism of action is too rapid to be produced by estrogen's classical actions on gene transcription.

In order to rule out the possibility that mER activation abolished ORL1 antinociception via direct or indirect involvement of genomic changes, anisomycin (125 μg) was administered 15-minutes prior to co-administration of E2BSA and OFQ (Fig. 1B). ANOVA revealed no significant main effect of group ($F_{(3, 10)}$ =1.42; p=0.29), time ($F_{(13,130)}$ =1.44; p=0.15), or interaction between group x time ($F_{(39,130)}$ =0.90; p=0.64) (Fig. 1B). Thus, anisomycin pretreatment in neither OVX (n=3) nor males (n=3) significantly altered the E2BSA-induced abolition of ORL1-mediated antinociception. Anisomycin itself did not alter baseline TFLs. These observations suggest that genomic mechanisms do not mediate the effects of E2BSA.

In a separate series of experiments, OVX females were co-administered doses of E2BSA with OFQ that mimicked low levels of estrogen during the diestrous phase of normally cycling female rats (0.09 nM; n=5); high levels of estrogen during the proestrous stage of normally cycling female rats (0.36 nM; n=6); and a maximal dose (0.5 mM; n=3) (Fig. 2). ANOVA yielded significant main effects of group ($F_{(2, 12)}$ =15.67; p=0.00), time ($F_{(13,143)}$ =7.76; p=0.00), and interaction between group x time ($F_{(26,143)}$ =3.41; p=0.00) (Fig. 2). *Post hoc* comparisons revealed that interaction stemmed from the fact that after three comparable baselines, 0.5 mM E2BSA +OFQ and 0.36 nM E2BSA+OFQ treated OVX groups had significantly higher tail flick latencies as compared to the 0.09 nM E2BSA+OFQ group. These data suggest that E2BSA attenuates ORL1-mediated antinociception in a physiologically relevant, dose-dependent manner.

To identify the relative contribution of each mER to the estrogen-induced attenuation of ORL1-mediated spinal antinociception, each mER was activated by selective agonists, as described below.

Activation of GPR30 attenuates ORL1-mediated spinal antinociception in OVX females and males

To assess the role of GPR30 in estrogen induced-attenuation of ORL-1 mediated spinal antinociception, OVX female and male rats were co-administered G-1 (0.25 nM) and OFQ (Fig. 3). ANOVA yielded significant main effects of group ($F_{(7, 39)}$ =15.94; p=0.00), time ($F_{(13,507)}$ =28.16; p=0.00), and interaction between group x time ($F_{(91,507)}$ =6.39; p=0.00) (Fig. 3A). *Post hoc* comparisons revealed that interaction stemmed from the fact that after three comparable baselines, veh+OFQ and G-15 treated groups had significantly higher tail flick latencies as compared to the rest of the groups. G-1 completely abolished ORL1-mediated antinociception in both OVX female (n=6) and male (n=8) groups. Pretreatment with G-15 (1 μM), GPR30 antagonist, 5 minutes prior to co-administration of G-1 and OFQ significantly increased TFLs in OVX females (n=4) and males (n=3), implicating a restoration of ORL1-mediated antinociception. Moreover, G-1+veh treatment did not alter TFLs in OVX (n=5) or males (n=4), suggesting that G-1 does not produce pronociceptive effects. Analysis of AUC yielded a significant main effect of group ($F_{(5, 46)}$ =5.53; p=0.00) (Fig. 3B) further confirming that AUCs in G-1 + OFQ and G-1+veh -treated groups were significantly lower than veh+OFQ and G-15-treated OVX and males. These data are consistent with the interpretation that activation of GPR30 contributes to the estrogen-induced attenuation of ORL1-mediated spinal antinociception.

Activation of Gq-coupled mER attenuates ORL1-mediated spinal antinociception in OVX females and males

To determine whether activation of Gq-mER mediates estrogen-induced attenuation of ORL1-mediated antinociception, STX (10 nM) and OFQ were co-administered and TFLs were recorded (Fig. 4). ANOVA yielded significant main effects of group ($F_{(5, 33)}$ =22.75; p=0.00), time ($F_{(13,429)}$ =20.46; p=0.00), and interaction between group x time ($F_{(65,429)}$ =6.80; p=0.00) (Fig. 4A). *Post hoc* comparisons revealed that interaction stemmed from the fact that after three comparable baselines, veh+OFQ treated OVX and male groups had significantly higher tail flick latencies as compared to the rest of the groups. STX significantly attenuated ORL1-mediated spinal antinociception in OVX female (n=8) and male (n=3) groups (Fig. 4A). STX+veh treatment did not alter TFLs in OVX (n=5) or males (n=4), indicating that STX does not produce pronociceptive effects. Analysis of AUC yielded a significant main effect of group ($F_{(5,38)}$ =22.82; p=0.00) (Fig. 4B), further confirming that AUCs were significantly lower in STX +OFQ and STX+veh-treated groups compared to OFQ-control groups. These data suggest that Gq-mER activation also contributes to estrogen –induced attenuation of ORL1-mediated antinociception.

Activation of ERα but not ERβ attenuates ORL1-mediated spinal antinociception in OVX females and males

A small percentage (5–10%) of cytosolic ERs (ERα and ERβ) is post-translationally palmitylated to enable ERs to be inserted into the plasma membrane of cells (Levin, 2011; Levin, 2009). In order to determine whether ERα contributes to the mER-induced attenuation of ORL1-mediated spinal antinociception, PPT (100 nM) was co-administered with OFQ and TFLs were recorded. As shown in Fig. 5A, ANOVA yielded significant main effects of group ($F_{(5, 27)}$ =44.16; p=0.00), time ($F_{(13, 351)}$ =10.77; p=0.00), and interaction between group x time ($F_{(65, 351)}$ =8.07; p=0.00) (Fig. 5A). *Post hoc* comparisons revealed that interaction stemmed from the fact that after three comparable baselines, veh+OFQ treated OVX and male groups had significantly higher tail flick latencies as compared to the rest of the groups. PPT significantly attenuated ORL1-mediated spinal antinociception in OVX (n=3) and males (n=4). PPT+veh treatment did not alter TFLs in OVX (n=3) or males (n=3), suggesting that PPT does not produce pronociceptive effects.

We conducted a similar experiment to determine whether ERβ activation also attenuated ORL1-mediated spinal antinociception (Fig. 5B). ANOVA yielded a significant main effects of group ($F_{(5, 23)}$ =26.33; p=0.00), time ($F_{(13, 299)}$ =34.03; p=0.00), and time x group interaction ($F_{(65, 299)}$ =4.78; p=0.00) (Fig. 5B). *Post hoc* comparisons revealed that interaction stemmed from the fact that after three comparable baselines, DPN+OFQ treated OVX and male groups had significantly higher tail flick latencies as compared to the rest of the groups. Thus, DPN has no effect on ORL1-mediated spinal antinociception in OVX females (n=3) or males (n=4). Furthermore, DPN + veh treatment did not alter TFLs in OVX (n=3) or males (n=3), suggesting that DPN does not produce antinociceptive effects. These observations suggest that activation of ERα but not ERβ contributes to the estrogen-induced attenuation of ORL1-mediated spinal antinociception.

Activation of mERs modulates levels of phosphorylated kinase in OVX females but not in males

To test the possible role of extracellular signal-regulated kinase 1 */2* (ERK 1 / 2), protein kinase A (PKA), or protein kinase C (PKC) in mediating mER activation-induced attenuation of ORL1 antinociception, spinal levels of total and phosphorylated kinase were measured after administering an intrathecal dose of E2BSA (0.5 mM; n=3), OFQ (n=3), or E2BSA+OFQ (n=3) and taking three TFL readings to confirm the expected antinociceptive or attenuated antinociceptive response. Densitometry of immunoblots revealed a significant

increase in pERK 1/2 levels (68.87%) in OVX females treated with E2BSA+veh compared to the OFQ control group (Fig. 6A) ($F_{(2, 8)}$ =6.06; p=0.04). When analyzed individually pERK1 in OVX showed no significant change regardless of treatment group (Fig. 6B, ($F_{(2, 8)}$) =1.337; p=0.33)); however, pERK2 increased 107.21% in E2BSA+OFQ-treated and 164.68 % in E2BSA+veh -treated OVX groups (Fig. 6C, ($F_{(2, 8)}$ =12.17; p=0.01)). Males showed no significant changes in pERK 1/2, pERK1, or pERK2 (Fig. 6D, ($F_{(2, 8)}$ =1.65; p=0.27); Fig. 6E, ($F_{(2, 8)}$) =1.63; p=0.27); Fig. 6F, ($F_{(2, 8)}$ =1.69; p=0.26)). *E2BSA* significantly decreased pPKA in the veh (78.32%) (p=0.00) and E2BSA +OFQ-treated (31.44%) (p=0.01) OVX groups compared to the OFQ control group (Fig. 7A, ($F_{(2, 8)}$ =24.16; p=0.00)). Males showed no significant changes in pPKA (Fig. 7B, ($F_{(2, 8)}$ =0.37; p=0.71)). pPKC was not altered by E2BSA in OVX (Fig. 7C, ($F_{(2, 8)}$ =2.14; p=0.20)) or males (Fig. 7D ($F_{(2, 8)}$ =0.88; p=0.46)) compared to OFQ control groups.

These observations suggest that ERK 1/2, specifically ERK2, but not PKA or PKC may play an important role in mediating mER –induced attenuation of ORL1antinociception in OVX females.

A causal role for ERK 1 /2 in mER-mediated attenuation of ORL-1 mediated spinal antinociception

To determine whether mER attenuation of ORL1-mediated spinal antinociception is dependent on phosphorylation of ERK 1 /2, OVX rats were pretreated with U0126 30 minutes prior to co-administration of E2BSA and OFQ and TFLs were recorded (Fig. 8). ANOVA revealed significant main effect of group ($F_{(1, 7)}$ =43.36; p=0.00), time ($F_{(13, 91)}$ =6.77; p=0.00), and time x group interaction ($F_{(13, 91)}$ =7.05; p=0.00) (Fig. 8). *Post hoc* comparison revealed interaction stemmed from the fact that after three comparable baselines, U0126 treated group had significantly higher tail flick latencies as compared to the U0124 treated group. Pretreatment with MEK/ERK inhibitor, U0126, (n=5) restored ORL1-mediated spinal antinociception; whereas, the inactive isomer, U0124, (n=4) had no effect on E2BSA-induced attenuation of ORL1-mediated spinal antinociception. Further, neither pretreatment with U0126 nor U0124 altered TFLs. These observations suggest that E2BSA-induced attenuation of ORL1-mediated spinal antinociception is ERK 1/2-dependent in OVX rats.

Discussion

To our knowledge, this study is the first to demonstrate that activation of mERs can rapidly attenuate opiate-induced spinal antinociception via an ERK-dependent, non-genomic mechanism. Thus, selective activation of GPR30 and Gq- mER attenuates ORL1-mediated spinal antinociception. We also noted that ERα but not ERβ attenuates ORL1-mediated spinal antinociception. Since blockade of de novo protein synthesis did not perturb estrogen-induced abolition of ORL1-mediated antinociception, we interpret these findings with agonists at the ERα receptor to be indicative of actions mediated via the subpopulation of ERα that is palmitylated (Levin, 2009) and thus accessible to membrane-impermeable ligand. These findings provide strong evidence that rapid and likely non-genomic effects of estrogen mediated via mERs play a significant role in the modulation of ORL1-mediated analgesia. In addition, we have previously shown that estrogen induces down regulation of the ORL1 gene expression in pro-estrous and estradiol treated OVX female rats (Flores et al., 2003) and abolishes ORLI-mediated antinociception in the trigeminal region (Flores et al., 2001), suggesting that genomic mechanisms may also contribute to estrogen-induced abolition of ORL1-mediated analgesia.

Although activation of mERs have been shown to initiate a host of intracellular signaling cascades in other systems (Kelly et al., 2002; Qui et al., 2003; Prossnitz et al., 2008), those

involved in mediating estrogen-induced regulation of pain and analgesia remain largely unknown. Our results demonstrate that activation of mERs enhances levels of pERK2 but not levels of either pPKA or pPKC at the time point examined (within 6 mins after E2BSA administration). Interestingly, these changes in pERK2 only are detected in the female. Importantly, an ERK inhibitor blocks the mER-induced abolition of ORL1- antinociception in the female, indicating that an ERK-dependent pathway contributes to estrogen-induced blockade of ORL1-mediated antinociception. Although, intracellular pathways that mediate estrogen-induced activation of ERK, independent of PKA and PKC, are not known in the spinal cord, estrogen is known to activate the Src- Ras-MEK-ERK pathway in cancer cell lines (Migliaccio et al., 1996; Migliaccio et al., 2003; Levin et al., 2011). Moreover, GPR30 has been shown to mediate estrogen-induced activation of G proteins that activate Src, an upstream activator of ERK in cancer cell lines (Prossnitz et al., 2008). A physical interaction between GPR30 and ERα has been also shown to occur in Ishikawa cells that is further enhanced in the presence of estrogen (Vivacqua et al., 2009; Albanito et al., 2008). Therefore, E2BSA would be expected to enhance such interaction in the present study. It is not known whether selective activation of ERα itself yields similar results. Nevertheless, our study reveals complete abolition of ORL1-mediated antinociception by selective or co-activation of ERα and GPR30 using PPT, G1 and E2BSA. GPR30 expression has also been shown to be up-regulated by epidermal growth factor (EGF) and tumor growth factor alpha (TGFα) in ERα-positive cancer cells (Vivacqua et al., 2009); however, these slow genomic changes are not expected to affect the present set of results.

ERK-dependent mechanisms that might lead to estrogen-mediated abolition of antinociception are shown in Figure 9. Among the downstream effectors of ORL1 receptors are G protein – gated inwardly rectifying potassium channels (GIRK) (Ikeda et al., 1997), leading to hyperpolarization of neurons. Of the four known subunits, only GIRK1 and GIRK2 are present in the spinal cord, and are exclusively localized in lamina II excitatory interneurons that receive prominent input from C fibers (Marker et al., 2006). Estrogen has been shown to rapidly uncouple μ opioid and $GABA_B$ receptors from GIRK channels in hypothalamic neurons, an effect blocked by inhibitors of PKA and PKC (Kelly et al., 1992; Kelly et al., 2003). Further, PKA- mediated phosphorylation of μ opioid receptor is known to decrease coupling of the μ opioid receptor to $G_{i/o}$ (Harada et al., 1990; Harada et al., 1989). Though we did not observe enhanced levels of pPKA or pPKC in our studies, our detection of estrogen-mediated increases in pERK makes it reasonable to postulate that ERK-dependent phosphorylation could similarly phosphorylate the ORL1 receptor and decrease its coupling to $G_{i/o}$, contributing to the mER-induced abolition of ORL1-mediated antinociception. It is known that ERK can phosphorylate voltage-gated potassium channels and decrease their conductance, thus enhancing neuronal excitability in the dorsal horn of the spinal cord (Adams et al., 2000; Hu et al., 2003; Ji et al., 1999; Ji et al., 2003; Schrader et al., 2006), which may represent another mechanism of relevance to our findings that an increase in phosphorylated ERK is a causal contributor to estrogen abrogation of ORL1-mediated spinal antinociception.

Relatively high levels of estrogen were able to attenuate ORL1-mediated analgesia in both males and OVX females. Although spinal synthesis of estrogen via aromatization of testosterone occurs in intact males (Liu et al., 2011), the amount of estrogen thus produced is unlikely to influence ORL1-induced analgesia since even a diestrous-equivalent dose of estrogen was ineffective in males (data not shown). Although there were insignificant behavioral differences between males and OVX females treated with mER agonists, no significant changes in kinase phosphorylation (PKA, PKC, or ERK1/2) were observed in the male, suggesting that sex-specific molecular mechanisms may be involved. This interpretation is consistent with our previous findings that ORL1-mediated antinociception by estrogen and testosterone is differentially regulated in the female and in the male

(Claiborne et al., 2006); estrogen abolishes antinociception in the female whereas testosterone is required for the expression of antinociception in the male. In addition, others have reported sexual dimorphism in PKA, PKCε and ERK modulation of alcohol neuropathy-induced mechanical hyperalgesia (Dina et al., 2007). Spinal GIRK2 expression has been shown to be sex hormone-dependent only in males (Ahangar et al., 2008) and therefore not likely to cause sex related differences in this study due to the inclusion of intact males. ERα ir-spinal neurons are shown to be significantly higher in intact male as compared to estrogen-treated female mice (Vanderhorst et al., 2005) suggesting that males would be more sensitive to ERα activation, however we did not observe a more robust effect of PPT in males. GPR30 distribution profiles are similar in male and female nervous system (Dun et al., 2009). Therefore, we suggest that mER-induced abolition of ORL1-mediated antinociception in the male might involve a male-specific, testosterone-dependent, and ERK – independent mechanism.

Although we did not observe a pronociceptive effect of E2BSA on tail flick assay in this study, it would be interesting to determine such effect of mER activation under different pain conditions such as inflammation or neuropathic pain. ORL1 receptor agonists show remarkable efficacy in alleviating hyperalgesia and allodynia associated with peripheral nerve injury and inflammation over other opioid agonists in rodents (Hayashi et al., 2010) and primates (Hu et al., 2010) implicating the promise of this molecular target for therapeutically managing pain control. Further, endogenous OFQ levels are increased in inflammatory pain states and OFQ knockout mice exhibit lower pain threshold than wild type mice, suggesting that OFQ acts as a physiological analgesic (Depner et al., 2003). Based on the conclusions of the present study it would be advantageous to determine if mER-induced rapid attenuation of ORL1-mediated antinociception could be pharmacologically circumvented by mER antagonists without interfering with important regulatory roles of estrogen in reproduction (Sinchak and Wagner, 2012), learning and memory (Spencer et al., 2008), and metabolism (Clegg, 2012).

It is noteworthy that supraspinal administration of OFQ produces pronociceptive effects (Grisel and Mogil, 2000; Wang et al. 1999; Grisel et al., 1996) with still unclear revelation of underlying mechanisms. However, an involvement of ERK kinase has been shown (Chen et al., 2008). In contrast, we have shown that intrathecal delivery of OFQ increases PKA phosphorylation in the female and activation of mERs attenuates OFQ-induced antinociception via an ERK2-dependent mechanism. Hence, distinct signaling mechanisms may underlie OFQ's contrasting effects when delivered spinally versus supraspinally and in males and females.

Lastly, identifying biological mechanisms by which estrogen abolishes opioid receptor-mediated spinal analgesia is critical to our understanding of how to improve therapeutic treatments for females suffering from chronic pain syndromes. Not only could estrogen-induced abolition of ORL1-mediated analgesia represent an important biological mechanism that makes women more susceptible to the development of certain pain syndromes, but the mERs identified in this study would represent useful targets for antagonism to overcome these syndromes in women.

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Highlights

- Activation of mERs attenuates ORL1- analgesia via a rapid non-genomic mechanism
- Selective activation of GPR30, Gq-coupled mER, ERα attenuate ORL1- analgesia
- Activation of mERs attenuate ORL1-analgesia via an ERK dependent mechanism in females

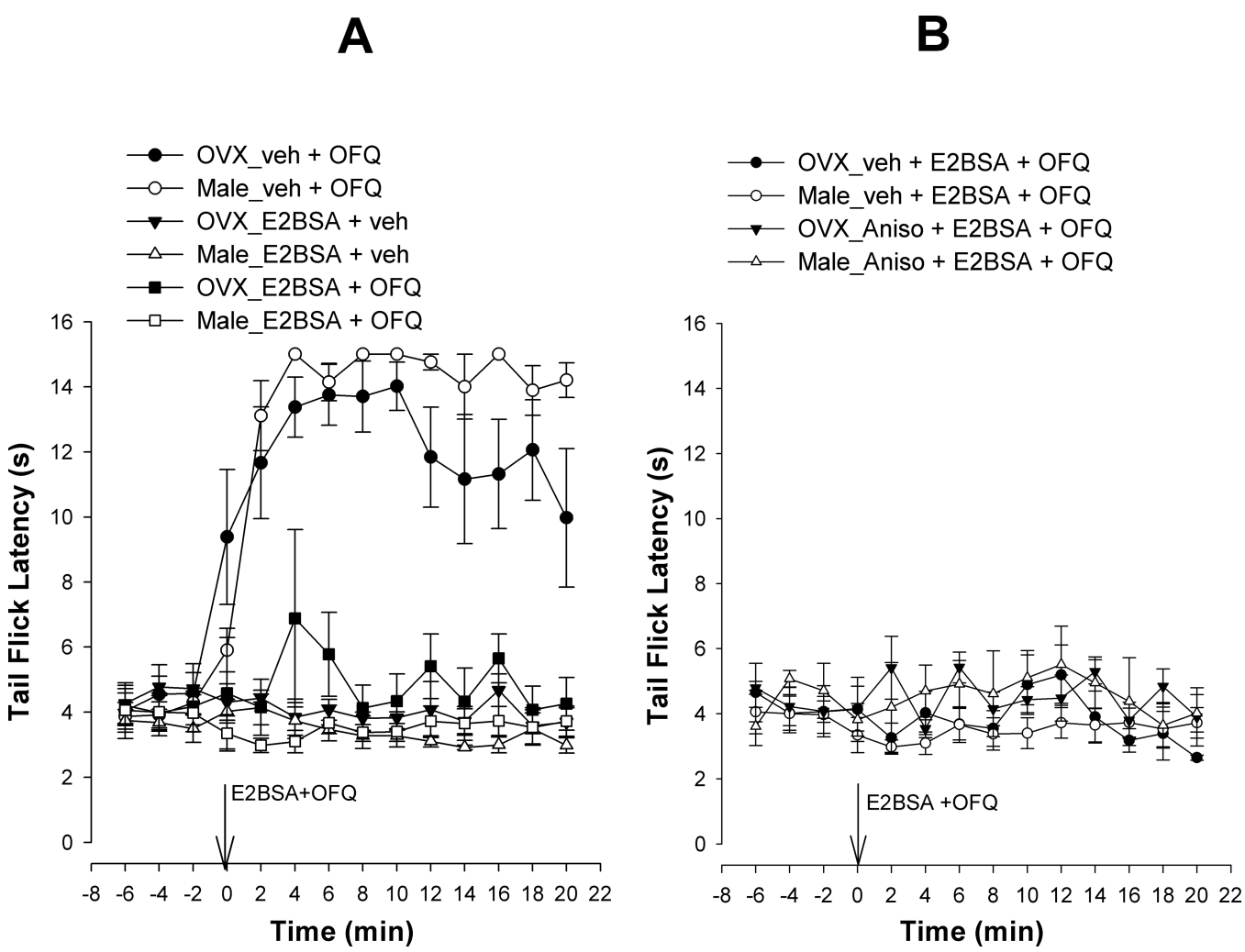

Figure 1.

Intrathecal administration of E2BSA rapidly attenuates OFQ-induced antinociception and does not involve de novo protein synthesis. **(A)** OFQ (10 nmol), given at time "0" significantly increased tail flick latencies (TFLs) in male and OVX rats. E2BSA (0.5 mM) abolished OFQ-induced increase in TFLs starting at "2 min" both in male and OVX rats. E2BSA + veh did not significantly change TFLs. **(B)** Anisomycin (125 μg), given intrathecally, 15 min prior to E2BSA+OFQ did not result in any significant change in E2BSA-induced attenuation of OFQ-induced TFL increase.

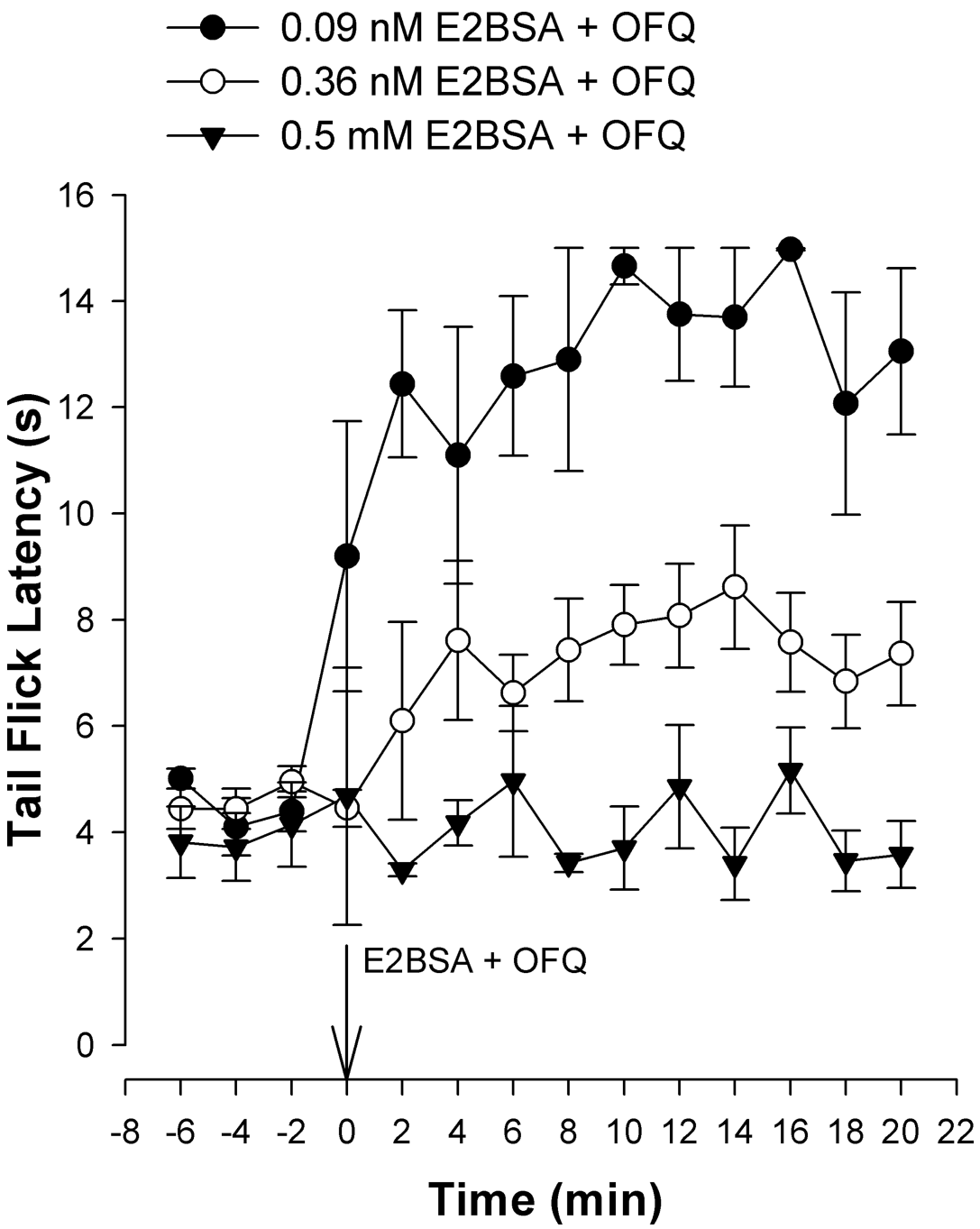

Figure 2.
Intrathecal administration of E2BSA dose-dependently attenuates OFQ-induced antinociception.

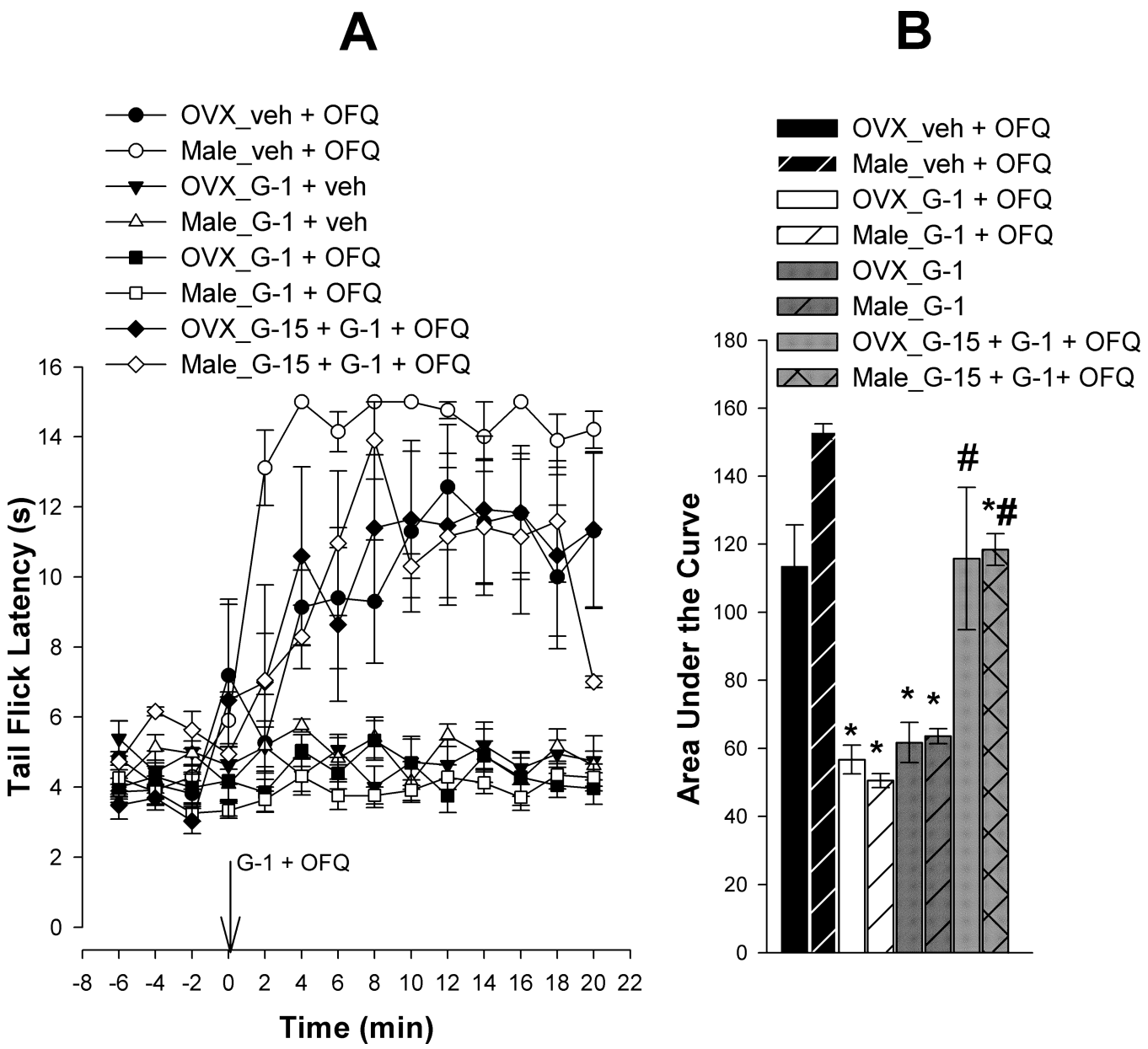

Figure 3.

Intrathecal administration of G-1 attenuates OFQ-induced antinociception. (**A**) G-1 (0.25 nM) significantly reduced OFQ-induced increase in TFLs within “4 min” in OVX and within “2 min” in male rats. G-15 (1 μM), given intrathecally 5 min prior to G-1 +OFQ restored the antinociceptive effect of OFQ in OVX and partially in males. G-1 +veh treatment did not significantly change TFLs. (**B**) AUC analysis of the data presented in (A) further confirmed these effects. G-1+OFQ and G-1+veh group AUCs were significantly lower than veh + OFQ and G-15 +G-1+OFQ groups. *p<0.05 as compared to respective veh +OFQ groups. #p<0.05 as compared to respective G-1+OFQ groups.

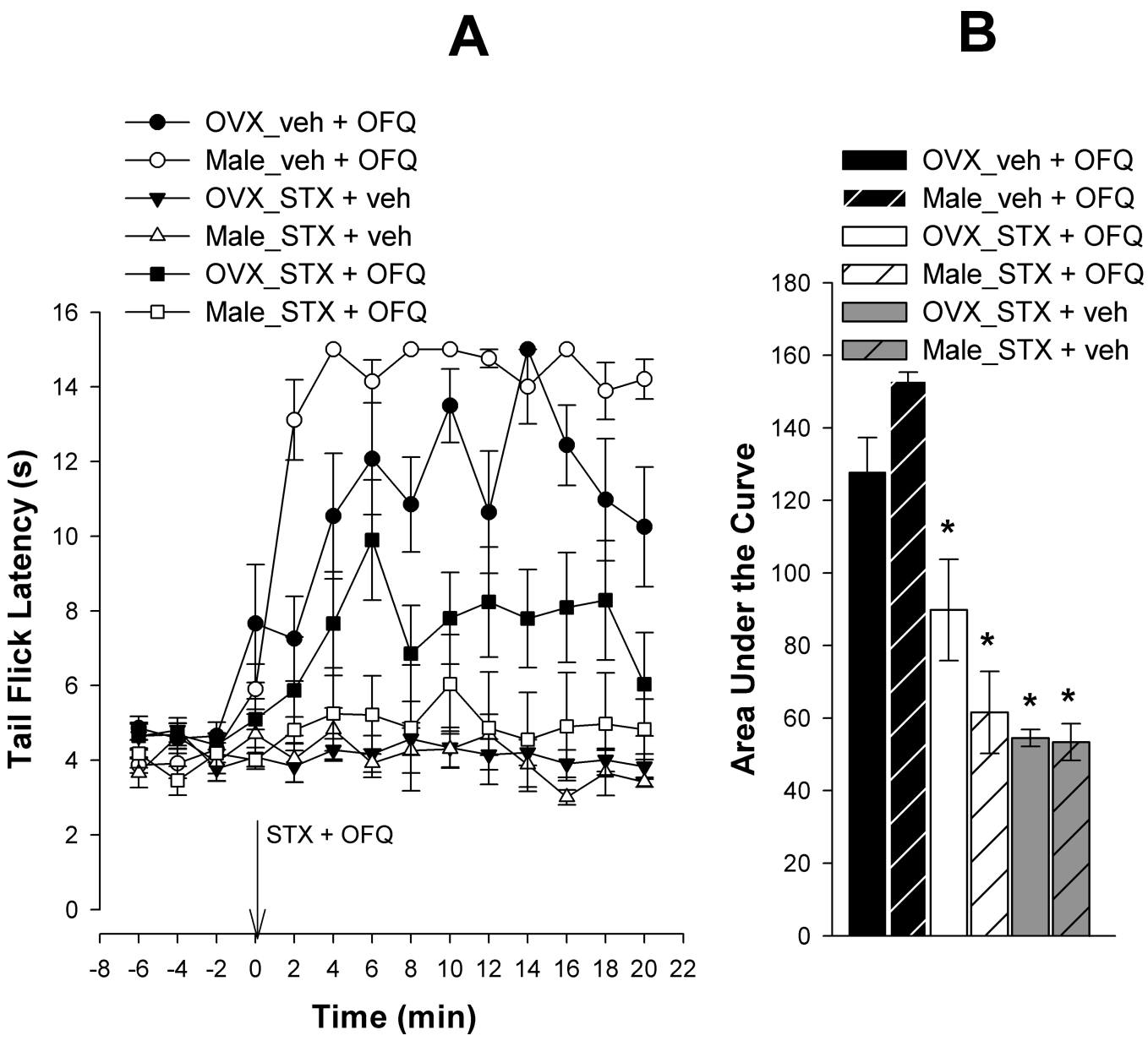

Figure 4.

Intrathecal administration of STX attenuates OFQ-induced antinociception. **(A)** STX (10 nM) significantly attenuated OFQ-induced increase in TFLs in both OVX and males. STX +veh -treated groups did not significantly change TFLs. (**B**) AUCs of STX+OFQ and STX + veh groups were significantly lower than AUCs of veh+OFQ groups in both OVX and males. *p<0.05 as compared to respective veh+OFQ -treated groups.

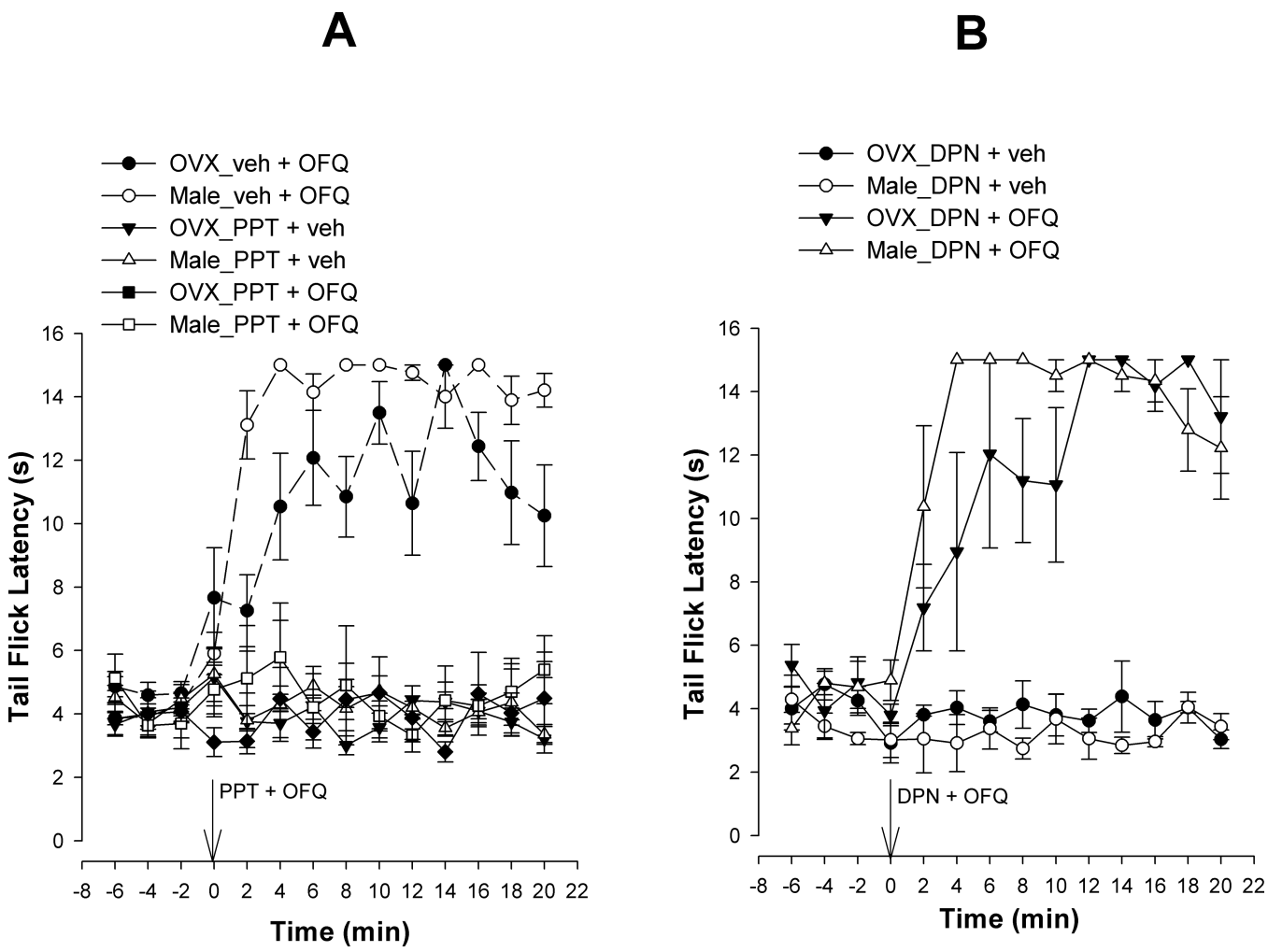

Figure 5.

Intrathecal administration of PPT, but not DPN attenuates OFQ-induced antinociception. (**A**) PPT (100 nM) significantly reduced OFQ-induced increase in TFLs in OVX and males. PPT+veh did not significantly change TFLs. Male and OVX groups treated with veh+OFQ are indicated by dashed lines (replotted from Fig.4A) for the sake of easy comparison. **(B)** DPN (100 nM) had no effect on OFQ-induced increase in TFLs in OVX and males. DPN +veh did not significantly change TFLs.

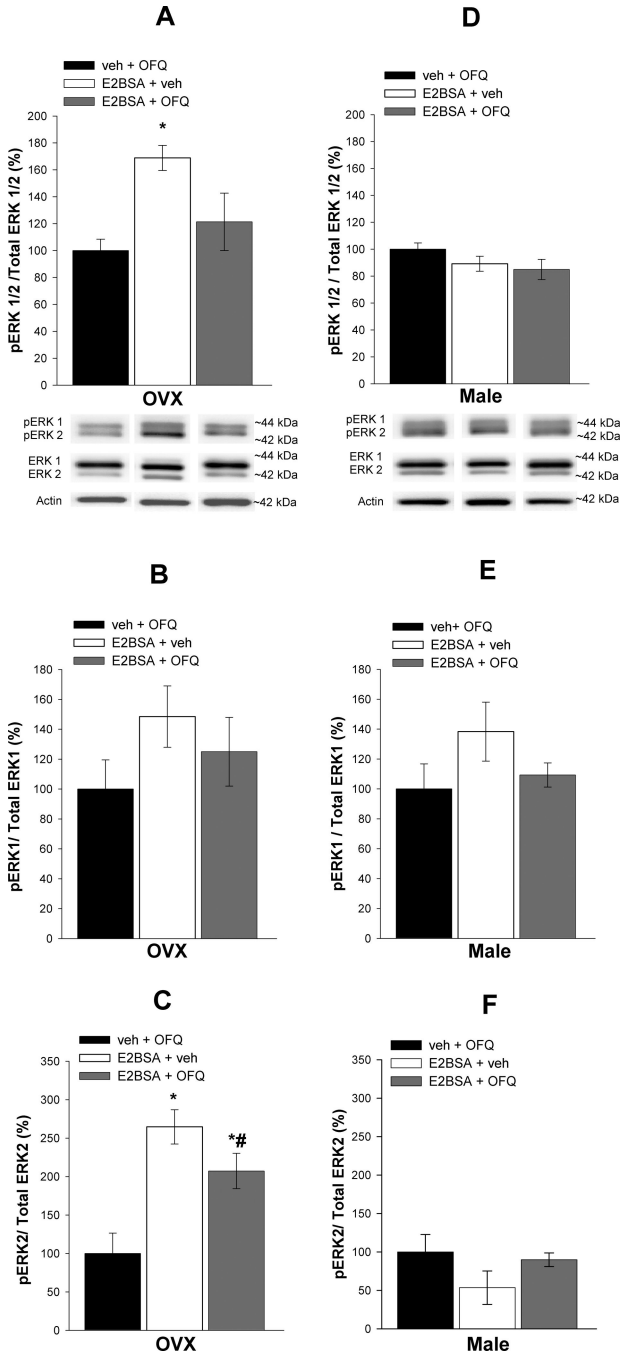

Figure 6.

Intrathecal administration of E2BSA significantly increases phosphorylation of ERK 1/2 in OVX, but not in males. Representative bands for each treatment group are shown at the bottom in panels (**A** and **D**). (**A**) Densitometric analysis showed that E2BSA+veh significantly increased levels of phosphorylated ERK 1/2 compared to the veh+OFQ group in OVX. When analyzed separately (ERK1 and ERK2), **(B)** E2BSA treatment showed no significant changes in phosphorylated ERK1 level in OVX, **(C)** but showed a significant increase in level of phosphorylated ERK2 in both E2BSA +OFQ and E2BSA+veh groups compared to the veh+OFQ group. E2BSA+OFQ group showed significantly less

phosphorylated ERK2 compared to the E2BSA+veh group. E2BSA had no effect on ERK1/2 phosphorylation in the male **(D–F)**. *p<0.01 as compared to respective veh+OFQ groups. #p<0.01 as compared to respective E2BSA+veh groups.

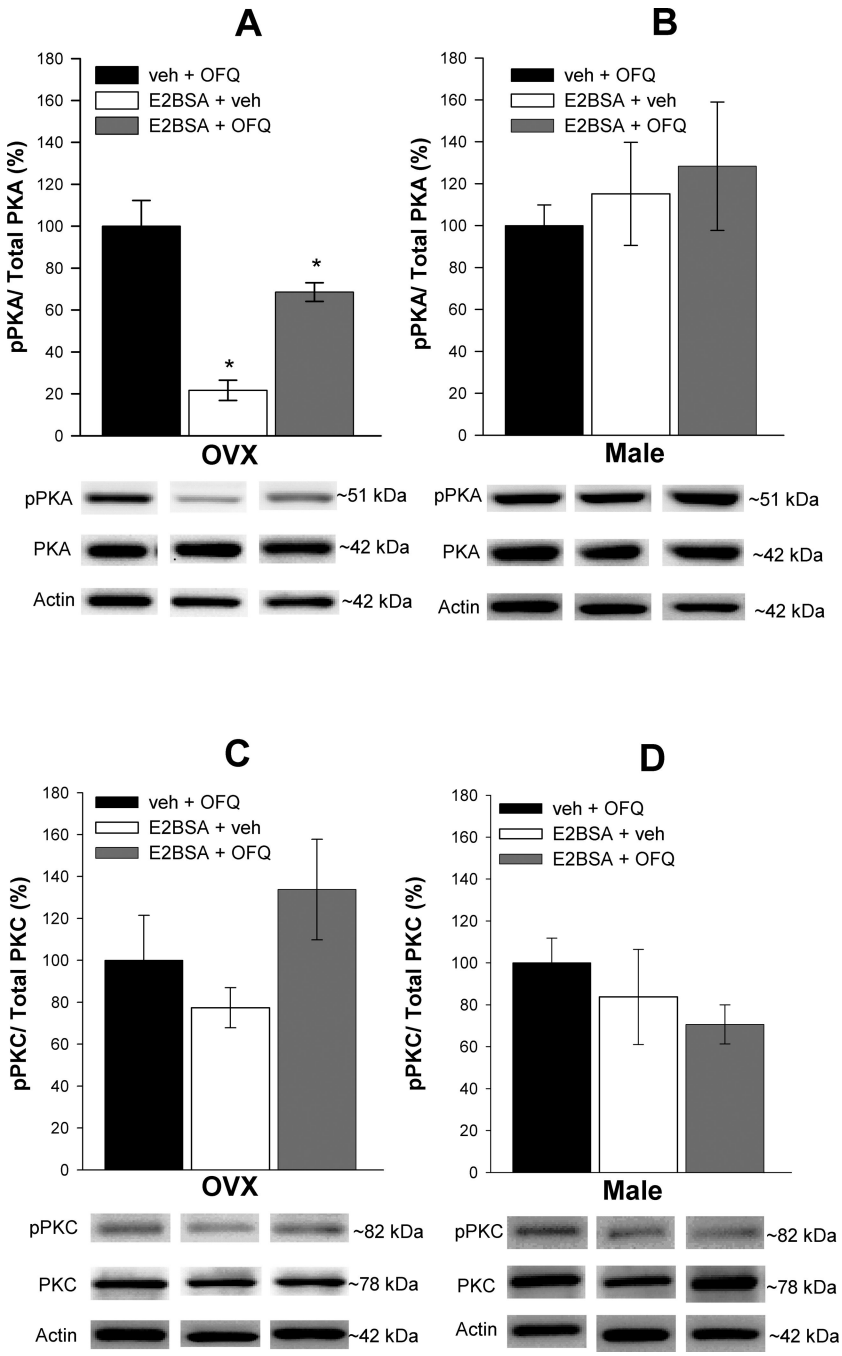

Figure 7.

Intrathecal administration of E2BSA decreases phosphorylation of PKA in OVX but not in males; and causes no change in phosphorylation of PKC in the rat spinal cord. Representative bands for each treatment group are shown at the bottom in panels (**A**-**D**). (**A**) Densitometric analysis showed that E2BSA significantly decreased phosphorylated PKA in E2BSA +OFQ and E2BSA+veh OVX groups compared to the veh+OFQ group, but had no effect in the male **(B)**. Further, E2BSA treatment had no effect on phosphorylated PKC protein levels in OVX **(C)** or males **(D)**. *p<0.01 as compared to respective veh+OFQ control groups.

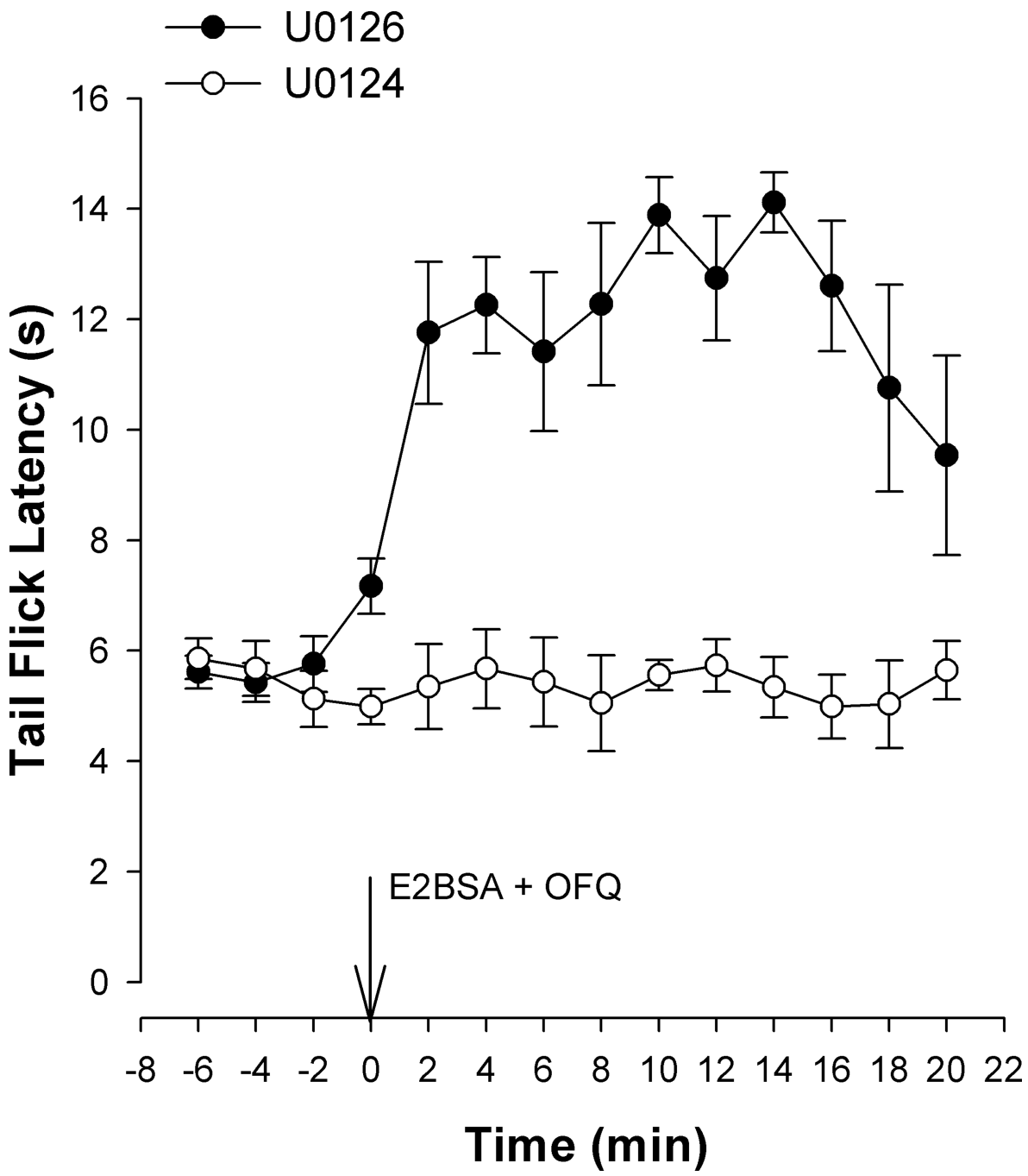

Figure 8.
Intrathecal administration of an ERK1/2 inhibitor, U0126 inhibits E2BSA-induced attenuation of ORL1-mediated antinociception. U0126 (10 μg), administered intrathecally 30 min prior to co-administration of E2BSA + OFQ restored the antinociceptive effect of OFQ. U0124 (10 μg), a control peptide, administered intrathecally 30 min prior to E2BSA +OFQ coadministration did not alter E2BSA-induced attenuation of OFQ-mediated antinociception.

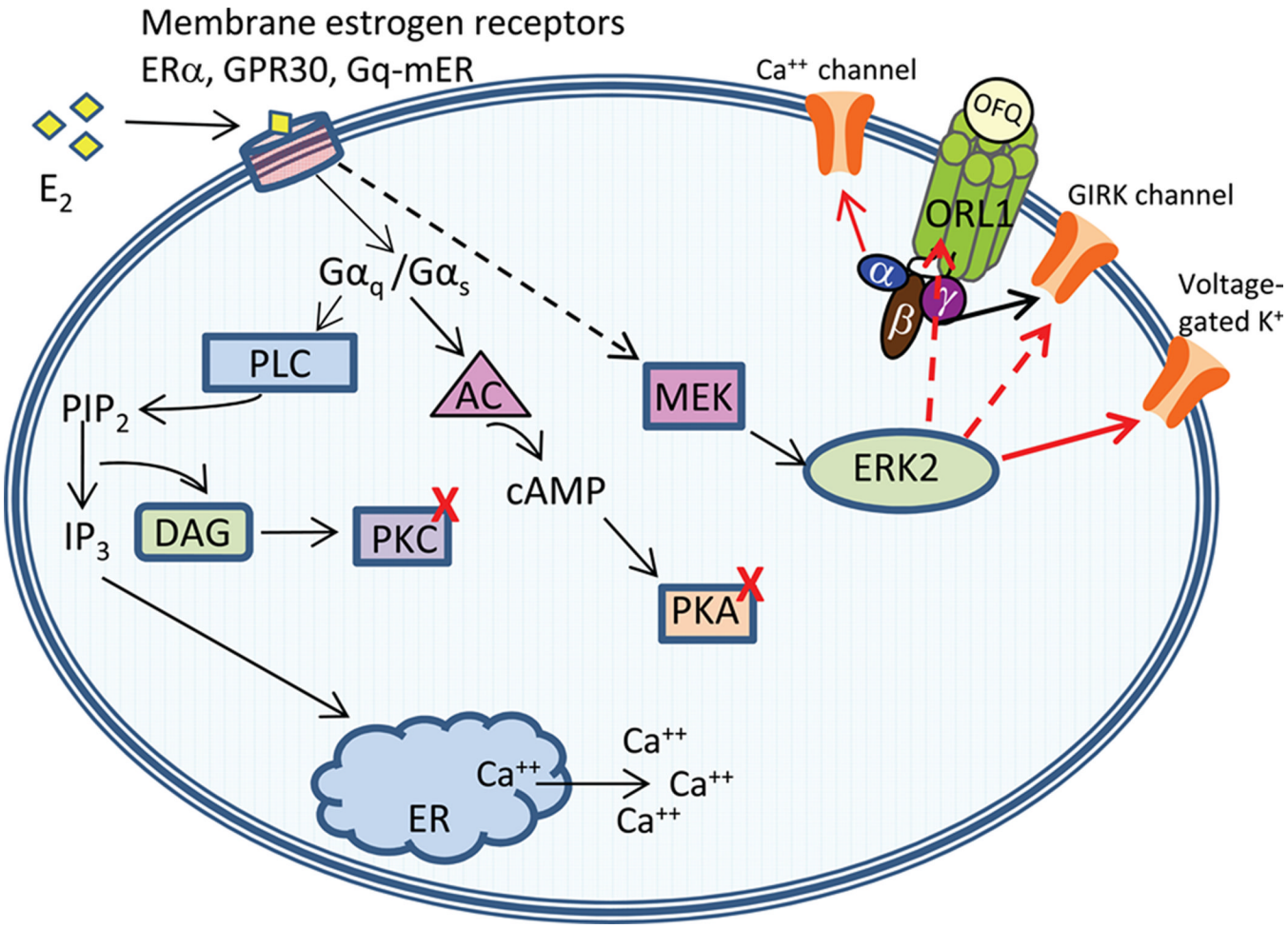

Figure 9.

Hypothesized schematic representation of the intracellular signaling cascade that mediates the mER-induced attenuation of ORL1 receptor- mediated spinal antinociception via rapid non-genomic mechanisms. Activated ORL1 receptor in the spinal cord couples to inhibitory G-proteins which decrease calcium channel conductance and facilitate receptor-operated potassium channel conductance to hyperpolarize neurons and produce analgesia. Estrogen-induced activation of mERs (GPR30, Gq-coupled mER, ERα, but not ERβ) non-genomically abolishes ORL1-mediated antinociception via (yet to be elucidated) intermediary signaling molecules that subsequently activate MAPK/ERK kinase (MEK) which phosphorylates (activates) ERK 2. ERK 2 can antagonize hyperpolarizing currents via one of the hypothesized mechanisms: (1) phosphorylate G-protein coupled inwardly rectifying potassium (GIRK) channels to decrease channel activity, (2) phosphorylate voltage-gated potassium channels to decrease their conductance and/or (3) phosphorylate the ORL1 receptor to decrease its coupling to inhibitory G-proteins resulting in decreased coupling of ORL1 to GIRK channels. We initially hypothesized mER activation-induced increase in PKA or PKC activation could potentially regulate hyperpolarizing currents via ERα and GPR30 activation coupling to $G\alpha_s$ proteins that activate adenyl cyclase (AC) which produces cAMP, the regulatory molecule for protein kinase A (PKA) activation; and Gq-mER coupling to $G\alpha_q$ proteins, which activate the phospholipase C (PLC) pathway to increase intracellular calcium and activate protein kinase C (PKC). However, our data suggests that mER activation abolishes ORL1-mediated spinal antinociception via a PKA

and PKC-independent mechanism. Taken together, activation of mER can decrease or abolish the ORL1-mediated hyperpolarization of dorsal horn neurons leading to reduction or abolition of ORL1-mediated analgesia. [red arrow-inhibition; black arrow -activation; dashed arrow - intermediate signaling molecules unknown] (PIP_2 -phosphatidylinositol 4, 5-bisphosphate, DAG- diacylglycerol, IP_3 -inositol 1,4,5-trisphosphate, Ca^{++} -calcium)