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## Estradiol-induced desensitization of 5-HT<sub>1A</sub> receptor signaling in the paraventricular nucleus of the hypothalamus is independent of estrogen receptor-beta

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### Summary

Estradiol regulates serotonin 1A(5-HT<sub>1A</sub>) receptor signaling. Since desensitization of 5-HT<sub>1A</sub> receptors may be an underlying mechanism by which selective serotonin reuptake inhibitors (SSRIs) mediate their therapeutic effects and combining estradiol with SSRIs enhances the efficacy of the SSRIs, it is important to determine which estrogen receptors are capable of desensitizing 5-HT<sub>1A</sub> receptor function. We previously demonstrated that selective activation of the estrogen receptor, GPR30, desensitizes 5-HT<sub>1A</sub> receptor signaling in rat hypothalamic paraventricular nucleus(PVN). However, since estrogen receptor beta(ER $\beta$ ), is highly expressed in the PVN, we investigated the role of ER $\beta$  in estradiol-induced desensitization of 5-HT<sub>1A</sub> receptor signaling. We first showed that a selective ER $\beta$  agonist, diarylpropionitrile(DPN) has a 100-fold lower binding affinity than estradiol for GPR30. Administration of DPN did not desensitize 5-HT<sub>1A</sub> receptor signaling in rat PVN as demonstrated by agonist-stimulated hormone release. Second, we used a recombinant adenovirus containing ER $\beta$  siRNAs to decrease ER $\beta$  expression in the PVN. Reductions in ER $\beta$  did not alter the estradiol-induced desensitization of 5-HT<sub>1A</sub> receptor signaling in oxytocin cells. In contrast, in animals with reduced ER $\beta$ , estradiol administration, instead of producing desensitization, augmented the ACTH response to a 5-HT<sub>1A</sub> agonist. Combined with the results from the DPN treatment experiments, desensitization of 5-HT<sub>1A</sub> receptor signaling does not appear to be mediated by ER $\beta$  in oxytocin cells, but that ER $\beta$ , together with GPR30, may play a complex role in central regulation of 5-HT<sub>1A</sub>-mediated ACTH release. Determining the mechanisms by which estrogens induce desensitization may aid in the development of better treatments for mood disorders.

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## Keywords

estrogen receptors; neuroendocrine responses; siRNA; recombinant adenovirus; serotonin receptors; mood disorders; estradiol

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## Introduction

Women past the age of 40 begin to experience fluctuation, then a decline in the levels of estrogens resulting in a peri- to post-menopausal state (Banger, 2002). Decreased levels of estrogens are associated with various neuropsychiatric disorders such as depression, anxiety, and panic disorders in women (Arpels, 1996). One particular hallmark of these disorders is a change in serotonergic function (Halbreich, 1990; Jimerson et al., 1997; Joffe and Cohen, 1998; Menkes et al., 1994; Ressler and Nemeroff, 2000), and particularly in serotonin 1A (5-HT<sub>1A</sub>) receptor function (Lemondé et al., 2003; Savitz et al., 2009; Shively and Bethea, 2004).

Several groups have shown that estrogen treatment modulates 5-HT<sub>1A</sub> receptor signaling. There is evidence for both species and regional differences in the regulation of 5-HT<sub>1A</sub> receptors following estrogen treatment. In rat studies, either a single injection or two-three days of estrogen treatment results in a decrease in 5-HT<sub>1A</sub> receptor gene expression (Osterlund and Hurd, 1998), G protein coupling (Mize and Alper, 2000), and 5-HT<sub>1A</sub> receptor function (D'Souza et al., 2004; Jackson and Uphouse, 1996, 1998; Raap et al., 2000) in the limbic region, cortex, and dorsal raphe nucleus. Similarly, chronic estrogen treatment in nonhuman primates and rats resulted in a decrease in 5-HT<sub>1A</sub> receptor gene expression (Birzniece et al., 2001), 5-HT<sub>1A</sub> receptor density, and 5-HT<sub>1A</sub> receptor-G protein coupling in the dorsal raphe nucleus (Lu and Bethea, 2002; Pecins-Thompson and Bethea, 1999). Following chronic estrogen treatment, 5-HT<sub>1A</sub> receptor gene expression (Birzniece et al., 2001) and 5-HT<sub>1A</sub> receptor-G protein coupling in cortical and hippocampal regions decreased without changes in 5-HT<sub>1A</sub> receptor density in rats (Le Saux and Di Paolo, 2005). In contrast, in nonhuman primates there was no evidence for changes in 5-HT<sub>1A</sub> receptor gene expression (Gundlah et al., 1999) or 5-HT<sub>1A</sub> receptor-G protein coupling in the hypothalamus (Lu and Bethea, 2002). The regional differences may be indicative of different estrogen receptor subtypes involved in the regulation of 5-HT<sub>1A</sub> receptors.

Understanding which estrogen receptor is involved in the regulation of hypothalamic 5-HT<sub>1A</sub> receptors may be important for improving the current therapies for mood disorders. Clinical studies have demonstrated that combining estrogen with SSRIs enhances the efficacy of the SSRIs in treating women with mood disorders and hot flashes (Schneider et al., 1997), but the mechanism by which this occurs is not known. Desensitization of somatodendritic 5-HT<sub>1A</sub> autoreceptors in the midbrain and postsynaptic 5-HT<sub>1A</sub> receptors in the hypothalamus are considered to be a likely underlying mechanism by which antidepressants, particularly SSRIs, mediate their therapeutic effects (Bosker et al., 2001; Chaput et al., 1986; Czachura and Rasmussen, 2000; Kreiss and Lucki, 1995). In contrast to SSRI administration which takes one-two weeks to produce a full desensitization (Li et al., 1997; Li et al., 1996), beta-estradiol 3-benzoate (EB) treatment results in partial desensitization of the 5-HT<sub>1A</sub> receptor signaling in the paraventricular nucleus of the hypothalamus (PVN) within 2 days (D'Souza et al., 2004; Raap et al., 2000).

Activation of the post-synaptic 5-HT<sub>1A</sub> receptors in the PVN stimulates the release of the hormones oxytocin, adrenocorticotropin hormone (ACTH), and corticosterone (Osei-Owusu et al., 2005). Based on our previous study of co-localization of estrogen receptors with 5-HT<sub>1A</sub> receptors, oxytocin and CRH, we know that 5-HT<sub>1A</sub> receptors co-localize with both

oxytocin and CRH in the PVN (Xu et al., 2008). Oxytocin is a neurohormone with anxiolytic and antidepressant activity and it is important in humans for socialization (Gimpl and Fahrenholz, 2001) whereas ACTH and corticosterone are pituitary and adrenal hormones respectively, associated with stress (Carrasco and Van de Kar, 2003). Measuring these hormones following activation of 5-HT<sub>1A</sub> receptors provides an indication of desensitization of 5-HT<sub>1A</sub> receptor activity in the PVN and can be used to assess the delay in the desensitization response to EB and other drug treatments.

We recently found that the G protein coupled estrogen receptor, GPR30, is involved in the desensitization of the 5-HT<sub>1A</sub> receptor signaling in the PVN (Xu et al., 2009). In addition to GPR30, PVN neurons also express the nuclear estrogen receptors, estrogen receptor alpha (ER $\alpha$ ) and beta (ER $\beta$ ). The density of ER $\beta$  is much higher than that of ER $\alpha$  in the PVN (Laflamme et al., 1998), especially in oxytocin neurons (Alves et al., 1998; Hrabovszky et al., 2004; Simonian and Herbison, 1997). Therefore in the current study, we focused on whether ER $\beta$  is involved in the EB-induced desensitization of the 5-HT<sub>1A</sub> receptors in the PVN. We used two complimentary approaches to address this question. In one experiment, we asked whether a selective ER $\beta$  agonist, diarylpropionitrile (DPN) desensitizes 5-HT<sub>1A</sub> receptors in the PVN. In a second complimentary experiment, we decreased the expression of the ER $\beta$  by injecting a recombinant adenovirus containing a small interference RNA (siRNA) against the ER $\beta$  into the PVN and then evaluated the effects of EB on 5-HT<sub>1A</sub> receptor signaling.

## Methods

### Animals

Female Sprague-Dawley rats (225–275g) from Harlan (Indianapolis, IN) were housed two per cage in a temperature-, humidity-, and light-controlled room (12h light/dark cycle, lights on 0700 h). Food and water were available ad libitum. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication 85-23, revised 1996) and as approved by the University of Kansas Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used.

### Drugs

EB was purchased from Sigma Research Biomedical Inc. (St. Louis, MO). DPN and (+)-8-hydroxy-2-dipropylaminotetralin hydrobromide ((+)-8-OH-DPAT) were purchased from Tocris (Ellisville, MO). (+)-8-OH-DPAT was dissolved in saline at a concentration of 0.2 mg/ml and administered at a dose of 0.2 mg/kg, sc. At this near maximal effective dose of (+)-8-OH-DPAT, the 5-HT<sub>1A</sub> receptor antagonist WAY-100635 blocks the hormone response by (+)-8-OH-DPAT suggesting that the effect is due to selective activation of 5-HT<sub>1A</sub> receptors over 5-HT<sub>7</sub> receptors at this dose (Critchley et al., 1994; Vicentic et al., 1998). EB was first dissolved in 100% ethanol to a concentration of 1 mg/ml and then diluted with sesame oil to a concentration of 5  $\mu$ g/ml. The EB solution and sesame oil were administered at 0.4 ml/kg (EB dose 2  $\mu$ g/kg, sc). DPN was first dissolved in 100% DMSO to a concentration of 40 mg/ml, and then this solution was diluted to 2 mg/ml with 25% DMSO/saline (administered at 1 ml/kg, sc). The DPN dose was chosen based on previous studies (Le Saux et al., 2006; Lund et al., 2005; Mazzucco et al., 2006). All of these solutions were made fresh before injection.

### Experiment 1: Effect of the selective estrogen receptor beta (ER $\beta$ ) agonist, DPN, on 5-HT $_1A$ receptor function

**DPN Binding to GPR30**—DPN is a selective ER $\beta$  agonist with an approximately 100-fold higher affinity for ER $\beta$  than estrogen receptor alpha (ER $\alpha$ ) (Lund et al., 2005). However, no data were available regarding the affinity of DPN for GPR30. Since GPR30 can mediate EB-induced desensitization of 5-HT $_1A$  receptors (Xu et al., 2009), it was necessary to determine the relative affinity of DPN for GPR30. DPN binding to human recombinant GPR30 was examined by a membrane estrogen radioreceptor assay described previously (Thomas et al., 2005). Plasma membranes of human recombinant GPR30-transfected HEK293 cells were prepared by homogenization with a glass homogenizer followed by sequential centrifugation steps of 1000 $\times$ g for 7 minutes and 20,000 $\times$ g for 20 min (Pang et al., 2008). An aliquot (0.125mg protein in 250 $\mu$ l) of the resulting plasma membrane pellet was incubated for 30 minutes with 2 nM of [ $^3$ H]-estradiol-17 $\beta$  ([ $^3$ H]-E2) in the presence of E2 or DPN at the concentrations 10 $^{-10}$ - 10 $^{-5}$  M. The bound [ $^3$ H]E2 was separated from free [ $^3$ H]E2 using glass-fiber filters. Competition binding was expressed as a percentage of maximum specific binding. The competitive binding assay was repeated three times.

**Effect of DPN treatment on 5-HT $_1A$  receptor function**—Rats were ovariectomized by removing both ovaries via a single ventral midline incision. Prior to surgery, an intraperitoneal injection of a mixture of ketamine hydrochloride (100 mg/kg) plus xylazine hydrochloride (10 mg/kg) was used to anesthetize the animals. Five days after surgery, animals were given subcutaneous (sc) injections of either DPN (2 mg/kg) or 25% DMSO/saline (vehicle) once a day for two days. A 5-HT $_1A$  receptor agonist, (+)8-OH-DPAT (0.2 mg/kg, sc) was injected 18 hours following the last injection of DPN or vehicle. Fifteen minutes later, animals were sacrificed by decapitation. Trunk blood was collected in centrifuge tubes containing 0.5 ml EDTA (0.3 M, pH 7.4). Plasma was stored at  $-80^{\circ}$ C until radioimmunoassays were conducted.

### Experiment 2: Effect of recombinant adenovirus containing ER $\beta$ siRNA on EB-induced desensitization of 5-HT $_1A$ receptors

**Generation of recombinant adenoviruses containing ER $\beta$  siRNAs**—To identify the potential siRNAs for ER $\beta$ , four ER $\beta$  siRNAs were selected for initial testing from a list of ER $\beta$  siRNAs suggested by a siRNA design program siDesign Center (Dharmacon, Inc., Thermo Fisher Scientific, Lafayette, CO). The sense sequences of the ER $\beta$  siRNAs were siRNA1:UCGCAAGUGUUAUGAAGUAUU; siRNA2:GCACAAGGAGUAUCUCUGUUU; siRNA3:AAUCAUCGCUCCUCUAUGCUU and siRNA4:GUCAAAGGUUCCGUGAGUUAUU. ER $\beta$  siRNA duplexes (Dharmacon, Inc., Thermo Fisher Scientific, Lafayette, CO) were transfected into PC-12 cells. The levels of ER $\beta$  mRNA in the cells were determined 48 hours later using RT-PCR.

Based on the extent of ER $\beta$  mRNA reduction, selected ER $\beta$  siRNAs were further evaluated using a pSOS-HUS vector (provided by Dr. TC He at the University of Chicago). The pSOS-HUS contains an siRNA site and a target gene site that allows transfection of siRNAs and the target gene into the same cells. The target gene is fused with an enhanced green fluorescent protein (GFP) gene, so that the expression of the target gene can be directly observed by fluorescence microscopy. The rat ER $\beta$  coding region was amplified by PCR with primers encoding 417–441 (Forward: ATGACATTCTACAGTCCTGTGTG) and 1874–1851 (reverse: TCACTGAGACTGTAGGTTCTGGG) of ER $\beta$  cDNA sequence (Accession # NM\_012754). The PCR product was then inserted into the target site of pSOS-HUS and fused with GFP (SOSER $\beta$ -HUS). After digestion with Sfi I at the siRNA insertion

site of SOS-ER $\beta$ -HUS, a double-stranded DNA oligonucleotide encoding ER $\beta$  siRNA1 or 2 sequences was ligated into SOS-ER $\beta$ -HUS to generate SOS-ER $\beta$ -siRNA-HUS.

The SOS-ER $\beta$ -siRNA-HUSs or SOS-ER $\beta$ -HUS was then transfected into HEK 293 cells to evaluate the knockdown efficiency of the ER-beta siRNAs. When comparing the SOS-ER $\beta$ -siRNA-HUSs transfected cells with those transfected with SOS-ER $\beta$ -HUS, a reduction in the number and density of GFP-containing cells indicates the efficiency of knockdown by the siRNA. In a separate experiment, the SOS-ER $\beta$ -siRNA-HUSs or SOS-ER $\beta$ -HUS was transfected into PC12 cells. 48 hours later, the cells were collected and lysed. The ER $\beta$  levels in the cell lysis were examined by western blot.

The siRNAs in the SOS-ER $\beta$ -siRNA-HUS that reduced the percentage and brightness of GFP-containing cells were selected and inserted into pSES-HUS vector (a shuttle vector for adenovirus). The SES-ER $\beta$ -siRNA-HUSs were further recombined into Ad-Easy-1 vector to generate high titer adenoviruses containing the siRNAs as described by Luo et al (He et al., 1998; Luo et al., 2007) and our previous publication (Li et al., 2004). Red fluorescent protein (RFP) is expressed independently along with the siRNAs by the adenoviral vector. RFP can be used as a marker for viral infection. The high titer ER $\beta$ -siRNA1-Ad and ER $\beta$ -siRNA2-Ad ( $\sim 10^{10}$  infectious viral particles/ml) were stored at  $-80^{\circ}\text{C}$ . To prevent tissue damage induced by the high salt storage-solution, the high titer recombinant adenovirus ER $\beta$ -siRNA-Ads (about 30  $\mu\text{l}$ ) were dialyzed with saline (about 1L) for at least 40 min at  $4^{\circ}\text{C}$  followed by 1:1 dilution with saline before use.

To evaluate ER $\beta$ -siRNA-Ads *in vivo*, the high titer ER $\beta$ -siRNA-Ads were unilaterally injected into the PVN using stereotaxic technique at a rate of 0.2  $\mu\text{l}/\text{min}$  (1  $\mu\text{l}/\text{side}$  at the coordinates of AP=  $-1.8$ , ML= 0.5 and DV=  $-8.3$  mm with respect to bregma (Paxinos and Watson, 2007). The needle was left at the site of injection for 20 minutes to reduce movement of the viral solution into the needle track. 7 and 10 days after the injection, rats were decapitated and the brains were collected to evaluate the efficiency of knockdown of ER $\beta$  expression by the siRNAs. Regions with viral infection as indicated by RFP and the contra-lateral regions (as controls) were punched for immunoblot analysis of ER $\beta$ .

#### **Effect of ER $\beta$ -siRNA-Ads on EB-induced desensitization of 5-HT $_{1A}$ receptors**

—After demonstrating that ER $\beta$  expression was decreased by the virus containing siRNAs for ER $\beta$ , the next experiment was to test the effect of ER $\beta$  knockdown on 5-HT $_{1A}$  receptor function. In this experiment, rats were anesthetized and ovariectomized as described above in Experiment 1. On the same day as the ovariectomy, either a control adenovirus (Ad-track, 1 $\mu\text{L}/\text{side}$ ) or ER $\beta$  siRNA-Ads (mixed ER $\beta$ -siRNA1-Ad and ER $\beta$ -siRNA2-Ad, 1 $\mu\text{L}/\text{site}$ ) was bilaterally microinjected into the PVN as described above. The control adenovirus (Ad-track) was generated by recombination of an empty Ad-track shuttle vector with the adenoviral vector.

On days 5 and day 6 after ovariectomy and viral injections, rats were injected with EB (2 $\mu\text{g}/\text{kg}$ , 0.4ml/kg) or sesame oil subcutaneously once a day for 2 days. To assess 5-HT $_{1A}$  receptor signaling in the PVN, the 5-HT $_{1A}$  receptor agonist, (+)8-OH-DPAT (0.2 mg/kg, sc) was injected 18 hours after the last EB or oil injection. Animals were sacrificed 15 minutes after the (+)8-OH-DPAT injection as described in **Experiment 1**. Trunk blood was collected for hormone radioimmunoassays. The brains were removed and flash-frozen in isopentane chilled over dry ice, and stored at  $-80^{\circ}\text{C}$  until further analysis.

Coronal brain sections were cut in a cryostat microtome to obtain a 700  $\mu\text{m}$  thick section at the level of the PVN (Paxinos and Watson, 2007). The PVN was dissected for western blot analysis of ER $\beta$  receptors. In addition, 16  $\mu\text{m}$  thick sections, before and after the 700  $\mu\text{m}$

PVN section, were collected and mounted on microscope slides. These sections were used to determine whether the adenovirus was correctly microinjected into the PVN.

### Immunoblot assay for ER $\beta$ receptors

The dissected brain tissues were homogenized in buffer (10 mM Tris, pH7.4, 100 mM NaCl, 1mM EDTA, 1 mM DTT, 0.1% sodium cholate and 1 $\times$  protease inhibitor cocktail) by sonication 5 sec  $\times$  3 time with 30 sec intervals in ice. After centrifugation at 25,000  $\times$  g, 4°C, the supernatant was used for western blots. The protein concentration was determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Rockford, IL).

ER $\beta$  levels in cell lysates or brain homogenates were measured using immunoblot analysis. The proteins (3  $\mu$ g/lane) were resolved on a 12% SDS-page gel followed by transferred to a PVDF membrane. The membranes were then incubated with blocking buffer (0.2% I-block (Tropix, Bedford, MA), 0.1% Tween 20 in Tris-buffered saline) followed by rabbit-anti ER $\beta$  (1:1000, Alexis Biochemicals, San Diego, CA) overnight at 4°C. The specificity of the ER $\beta$  antibody was demonstrated in our experiments in which a single band (~48 KD) was detected on western blots. The intensity of the 48 KD band was increased with over-expression in PC12 cells (Figure 3D), and no bands were detected in U-2 OS cell lysates (Figure 3B), a cell line that does not express ER $\beta$  (Levy et al., 2009). Moreover, the size of the band detected in PC12 cells was the same as in the PVN (Figure 3C). After washes, the membranes were incubated with a HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, 1:10,000). The bands were detected with ECL substrate solution (GE Healthcare Biosciences Corp, Piscataway, NJ) on x-ray film. The membranes were washed and then incubated with monoclonal mouse actin antibody (MP Biomedicals, Solon, OH, 1:10,000) and processed for the detection of actin immunoreactivity.

Films from the western blots were analyzed densitometrically using MCID Basic Program version 7.0 (Interfocus Imaging Ltd., Cambridge, England). The integrated optical density of protein bands were calculated as previously described (Raap et al., 2000). The density of the ER $\beta$  bands was normalized to the density of the  $\beta$ -actin band in the same sample. The percent reduction of ER $\beta$  expression induced by ER $\beta$ -siRNA-Ads during preliminary testing was calculated by comparing ER $\beta$  expression on the side injected with virus to the contralateral side. To determine the effect of ER $\beta$ -siRNA-Ads on EB-induced desensitization of 5-HT $_1A$  receptors, ER $\beta$  expression was normalized to actin expression in the sample, then divided by the mean of the control animals for each immunoblot and expressed as a percent mean of the control samples.

### Radioimmunoassay of plasma oxytocin and ACTH

Plasma oxytocin was determined by a radioimmunoassay as previously described with minor modifications (Li et al., 1997). Briefly, oxytocin was extracted from 0.5 mL plasma with 1 mL cold acetone followed by 2.5 mL of petroleum ether (Li et al., 1997). The ether layer (top layer) was aspirated and the samples were dried in a Centrivap vacuum concentrator at 4°C. The dried oxytocin residue was resuspended in 1mL of cold assay buffer (0.05M phosphate buffer pH7.4 containing 0.125% bovine serum albumin and 0.001M EDTA). The plasma extract (100 or 300  $\mu$ L) was used for the radioimmunoassay as previously described. The radioactive  $^{125}$ I oxytocin (specific activity: 2200 Ci/mmol) was obtained from Perkin Elmer (Waltham, MA). Several standard recovery samples containing 0.5 ml pooled plasma and 8 pg or 16 pg oxytocin were included through the extraction and assay procedure. The plasma oxytocin was calculated based on the recovery and dilution factors. Plasma ACTH concentrations were determined by radioimmunoassay as previously described (Li et al., 1993).

## Statistical analysis

All data are expressed as means  $\pm$  SEM. Oxytocin and ACTH data were analyzed by a two-way analysis of variance (ANOVA) for the DPN experiments or three-way ANOVA for the estrogen siRNA viral injection experiments and a Student-Newman Keuls post hoc test (Statview version 5.0 software, SAS Institute Inc., Cary, NC). Animals with misplaced injections or that were injected with the siRNA construct for ER $\beta$  but did not have a decrease in ER $\beta$  expression were excluded from the data analysis.

## Results

### Experiment 1: Effect of selective estrogen receptor beta (ER $\beta$ ) agonist, DPN, on 5-HT $_1A$ receptor function

**DPN has a low affinity for GPR30**—To determine the affinity of DPN for GPR30, we conducted a competitive  $^3H$ -E2 binding assay using cell membranes prepared from HEK293 cells transfected with human GPR30. As shown in figure 1, E2 is able to completely inhibit the  $^3H$ -E2 binding to GPR30 with a  $K_i \sim 10^{-7}M$ . The competition curve of DPN was shifted to the right relative to E2. The  $^3H$ -E2 binding was not completely blocked even with  $10^{-5}M$  of DPN. These results suggest that the affinity of DPN to GPR30 is at least 100 fold lower than E2.

**Effect of DPN treatment on 5-HT $_1A$  receptor function**—Two days of treatment with the ER $\beta$  agonist, DPN, did not alter the baseline levels of plasma oxytocin in comparison to the vehicle-treated animals (Figure 2A). Activation of 5-HT $_1A$  receptors by (+)8-OH-DPAT increased plasma oxytocin levels as expected, but DPN treatment did not alter this increase (Figure 2A). The two-way ANOVA for plasma oxytocin indicated a significant effect of (+)8-OH-DPAT injection ( $F_{(1, 35)} = 218.7, p < 0.0001$ ), but no significant effect of DPN treatment ( $F_{(1, 35)} = 0.13, p = 0.72$ ) and no significant interaction between DPN treatment and (+)8-OH-DPAT injection ( $F_{(1,35)} = 0.095, p = 0.76$ ).

Baseline plasma ACTH levels were not different in animals treated with DPN in comparison to the vehicle-treated animals (Figure 2B). As expected, 5-HT $_1A$  receptor activation by (+)8-OH-DPAT increased plasma ACTH levels, but DPN treatment had no effect on the (+)8-OH-DPAT-induced increase in the plasma ACTH (Figure 2B). The two-way ANOVA for plasma ACTH indicated a significant effect of (+)8-OH-DPAT injection ( $F_{(1, 35)} = 304.7, p < 0.0001$ ), but no significant effect of DPN treatment ( $F_{(1, 35)} = 0.45, p = 0.51$ ) and no significant interaction between DPN treatment and (+)8-OH-DPAT injection ( $F_{(1,35)} = 0.48, p = 0.5$ ).

### Experiment 2: Effect of recombinant adenovirus containing ER $\beta$ siRNA on EB-induced desensitization of 5-HT $_1A$ receptors

**Selection and evaluation of ER $\beta$ -siRNA-Ads**—Of the four ER $\beta$  siRNAs tested in PC12 cells, siRNA1 and 2 reduced ER $\beta$  mRNA more than 75%, whereas the siRNA3 and 4 only reduced ER $\beta$  mRNA about 20% (data not shown). Therefore, we selected the ER $\beta$  siRNA1 and 2 for further analysis. SOS-ER $\beta$ -siRNA-HUSs and SOS-ER $\beta$ -HUS were generated and transfected into HEK 293 cells. As shown in Figure 3A, the number and brightness of GFP-containing cells in SOS-ER $\beta$ -siRNA-HUS-transfected cells is significantly lower than that in SOS-ER $\beta$ -HUS-transfected cells. Consistent with these results, we also found that ER $\beta$  in the SOS-ER $\beta$ -siRNA-HUS-transfected cells was reduced relative to the SOS-ER $\beta$ -HUS-transfected cells using an immunoblot assay (Fig. 3D). Thus, the ER $\beta$  siRNA1 and 2 were selected for production of ER $\beta$  siRNA recombinant adenovirus.

To test whether high titer ER $\beta$ -siRNA-Ads are able to reduce the ER $\beta$  expression in vivo, we unilaterally injected the high titer ER $\beta$ -siRNA-Ads into the PVN. As shown in Figure 3E, the ER $\beta$  levels in the ER $\beta$ -siRNA-Ad injected sides were lower than that in the contralateral uninjected sides. The percent of inhibition was within the range of 47% to 81% (n=5). The reduction of ER $\beta$  expression was slightly larger seven days than ten days after viral injection (data not shown).

**ER $\beta$  in PVN following ER $\beta$ -siRNA-Ads injection and EB treatment**—Viral injections were monitored by the presence of RFP in the PVN as shown for one animal in Fig. 3F. Animals with incorrect injections were not included in subsequent analyses. We used both the ER $\beta$  levels in the PVN and the location of RFP expression as exclusion criteria for “incorrect injections” of ER $\beta$ -siRNA-Ads. Rats that did not express RFP in the PVN were excluded first (< 10%). We also excluded the rats for which the reduction of ER $\beta$  was less than 20%. The overall rate of successful injection was ~75%. Among rats with incorrect injections, the hormone responses to 8-OH-DPAT were similar to those Ad-track viral injected rats. No changes in the basal hormone levels or other behaviors were observed in the rats with incorrect injections.

Alterations in ER $\beta$  after EB treatment and/or ER $\beta$ -siRNA-Ads injection were measured using immunoblot analysis (Figure 4). Two days of EB treatment decreased ER $\beta$  protein levels in the PVN by 33% in animals microinjected with the control adenovirus. Microinjection with the ER $\beta$ -siRNA-Ads resulted in 34% decrease in ER $\beta$  protein levels. In animals treated with both ER $\beta$ -siRNA-Ads and EB, ER $\beta$  protein levels decreased by 50% in the PVN. The two-way ANOVA for ER $\beta$  protein levels indicated a significant effect of the viral injection ( $F_{(1, 34)} = 18.1, p = 0.0002$ ) and significant effect of EB treatment ( $F_{(1, 34)} = 16.9, p = 0.0002$ ), but no significant interaction between the viral injection and EB treatment ( $F_{(1,34)} = 1.81, p=0.19$ ).

**Effect of ER $\beta$ -siRNA-Ads on EB-induced desensitization of 5-HT $_1A$  receptors**—There was no difference in the baseline levels of plasma oxytocin in control virus-injected or ER $\beta$ -siRNA-Ads-injected animals (Figure 5A). (+)8-OH-DPAT increased plasma oxytocin levels in the control viral-injected and ER $\beta$ -siRNA-Ads-injected groups. EB treatment decreased the oxytocin response to (+)8-OH-DPAT by 28% and 30% in comparison to the oil-treated animals in the control viral-injected and ER $\beta$ -siRNA-Ads-injected animals, respectively (Figure 5A). The injection of ER $\beta$ -siRNA-Ads did not alter the effects of EB treatment on (+)8-OH-DPAT-stimulated oxytocin release in comparison to the control virus- injection. The three-way ANOVA for plasma oxytocin indicated a significant effect of (+)8-OH-DPAT injection ( $F_{(1, 40)} = 220.85, p<0.0001$ ), EB treatment ( $F_{(1, 40)} = 10.386, p=0.0025$ ), and a significant interaction between EB treatment and (+)8-OH-DPAT injection ( $F_{(1,40)} = 6.986, p = 0.01$ ). However, there was no significant effect of viral injection ( $F_{(1, 40)} = 0.072, p= 0.79$ ) and no significant interaction between the viral injection and EB treatment ( $F_{(1,40)} = 0.191, p = 0.66$ ), viral injection and (+)8-OH-DPAT injection ( $F_{(1,40)} = 0.007, p = 0.93$ ), or viral injection, EB treatment, and (+)8-OH-DPAT injection ( $F_{(1,40)} = 0.045, p = 0.83$ ).

Baseline levels of plasma ACTH were not different in control viral-injected or ER $\beta$ -siRNA-Ads-injected animals. In the control viral-injected animals, (+)8-OH-DPAT increased plasma ACTH levels. The effect of (+)8-OH-DPAT was decreased 23% by EB treatment (Figure 5B). In the ER $\beta$ -siRNA-Ads injected animals, the ACTH response to (+)8-OH-DPAT in oil-injected animals was 27% lower than that in the control virus/oil-injected animals (Figure 5B). In the ER $\beta$ -siRNA-Ads-injected animals, EB treatment resulted in a 43% increase in the plasma ACTH response to (+)8-OH-DPAT in comparison to the ER $\beta$ -siRNA-Ads/oil-injected animals (Figure 5B). The three-way ANOVA for plasma ACTH



indicated a significant effect of EB treatment ( $F_{(1, 46)} = 4.29$ ,  $p < 0.05$ ), (+)8-OH-DPAT injection ( $F_{(1, 46)} = 390.18$ ,  $p < 0.0001$ ), significant interaction between viral injection and EB treatment ( $F_{(1,46)} = 22.41$ ,  $p < 0.0001$ ), and significant interaction between the viral injection, EB treatment, and (+)8-OH-DPAT injection ( $F_{(1,46)} = 20.11$ ,  $p < 0.0001$ ). There was no significant effect of the viral injection ( $F_{(1, 46)} = 2.1$ ,  $p = 0.16$ ), no significant interaction between the viral injection and (+)8-OH-DPAT injection ( $F_{(1,46)} = 1.741$ ,  $p = 0.19$ ), or between the EB treatment and (+)8-OH-DPAT injection ( $F_{(1,46)} = 3.11$ ,  $p = 0.08$ ).

## Discussion

Previously, we reported that peripheral administration of EB or intra-PVN injections of G-1, a selective GPR30 agonist, for two days resulted in desensitization of 5-HT<sub>1A</sub> receptor signaling in the PVN (D'Souza et al., 2004; Raap et al., 2000; Xu et al., 2009). In the current study, we found that EB-mediated desensitization of the 5-HT<sub>1A</sub> receptor signaling in the PVN is independent of ER $\beta$ . However the effects of estradiol on ACTH release are more complex.

To determine whether ER $\beta$  is involved in the EB-induced desensitization of 5-HT<sub>1A</sub> receptor signaling in the PVN, we first examined the effects of ER $\beta$  agonist, DPN, on neuroendocrine responses to 5-HT<sub>1A</sub> receptor activation. Since treatment with GPR30 agonists result in desensitization of 5-HT<sub>1A</sub> receptor signaling (Xu et al., 2009) and no data were available concerning the affinity of DPN for GPR30, we determined the affinity of DPN for GPR30. We found that DPN has a 100 fold lower affinity for the GPR30 receptor in comparison to EB. Thus, DPN could be used to selectively stimulate ER $\beta$ . We found that DPN administration for two days did not alter the 5-HT<sub>1A</sub> receptor-stimulated release of oxytocin and ACTH. The dose of DPN (2 mg/kg) used in the current experiments is comparable to an effective dose used by other investigators in female rats to activate ER $\beta$  as demonstrated by decreases in anxiety behaviors, regulation of dopamine-2 receptors, and increases in cell proliferation (Le Saux et al., 2006; Lund et al., 2005; Mazzucco et al., 2006). Therefore, the present results suggest that activation of ER $\beta$  may be not involved in the desensitization of the 5-HT<sub>1A</sub> receptor signaling in the PVN. We used the same number of doses and timepoints for administration of DPN that we used for EB administration in order to determine if EB was working via ER $\beta$ . However, it is possible that other time points for administration of DPN would result in desensitization of 5-HT<sub>1A</sub> receptor signaling in the PVN.

To further evaluate the contribution of ER $\beta$  to the EB-mediated desensitization of the 5-HT<sub>1A</sub> receptor signaling, an adenovirus containing siRNA sequences against ER $\beta$  (ER $\beta$  siRNA-Ads) was directly injected into the PVN. Treatment with the ER $\beta$  siRNA-Ads decreased the protein levels of ER $\beta$  in the PVN by 34%. On the other hand, administration of EB for two days reduced the protein level of ER $\beta$  in the PVN by a comparable amount, 33%. This is consistent with the findings from other investigators who found that three to seven days of treatment with EB decreases ER $\beta$  mRNA and protein expression in the PVN (Patisaul et al., 1999; Suzuki and Handa, 2004). We found that EB administration regulates ER $\beta$  protein levels in the PVN to a similar extent as our ER $\beta$  siRNA-Ads injections. The combined treatment with both EB and ER $\beta$  siRNA-Ads resulted in a 50% decrease in the protein levels of ER $\beta$  in the PVN.

As we previously observed, two days of EB treatment desensitized 5-HT<sub>1A</sub> receptor signaling in oxytocin cells in the PVN (D'Souza et al., 2004; Raap et al., 2000). It appears that ER $\beta$  does not mediate the EB-induced desensitization of the 5-HT<sub>1A</sub> receptor signaling in oxytocin cells in the PVN based on the results from both experiments. First, treatment with the selective ER $\beta$  agonist DPN for two days did not desensitize 5-HT<sub>1A</sub> receptor

signaling. Secondly, decreasing the ER $\beta$  levels in the PVN did not alter the effects of EB on 5-HT $_{1A}$  receptor agonist-stimulated release of oxytocin. The oxytocin neurons express very little ER $\alpha$  (Simonian and Herbison, 1997), thus this receptor is unlikely to contribute to the desensitization of the 5-HT $_{1A}$  receptors. However, studies to directly address the role of ER $\alpha$  in EB-mediated desensitization of 5-HT $_{1A}$  receptor signaling are needed to unequivocally resolve the question. Taken together with our previous findings that two days of treatment with the selective GPR30 agonist G-1 induces desensitization of 5-HT $_{1A}$  receptors (Xu et al., 2009), we propose that GPR30 mediates the EB-induced desensitization of the 5-HT $_{1A}$  receptor signaling in oxytocin cells.

EB-induced regulation of 5-HT $_{1A}$  receptor signaling in CRH-containing cells in the PVN, measured using ACTH as the peripheral marker for central activation of CRH neurons, is more complex. EB treatment has not been shown to consistently desensitize 5-HT $_{1A}$  receptor-mediated ACTH release (D'Souza et al., 2004; Raap et al., 2000). In the current experiment, desensitization of the 5-HT $_{1A}$  receptor signaling in CRH-containing cells was observed in rats treated with the control virus and EB. Based on our DPN data, it would appear that ER $\beta$  is not directly involved in the desensitization of the 5-HT $_{1A}$  receptor signaling in CRH cells. However, decreasing ER $\beta$  in the PVN differentially altered the ACTH response to 5-HT $_{1A}$  receptor activation in oil and EB treated rats. Recent reports suggest that estrogens and their receptors regulate CRH expression and release via complex mechanisms (Solomon and Herman, 2009). The ACTH response to 5-HT $_{1A}$  receptor activation in ER $\beta$ -siRNA-Ad injected rats may be mediated by different mechanisms in the absence and presence EB. We speculate that the decrease in ACTH release in the ER $\beta$ -siRNA-Ads/oil treated animals could be due to ER $\beta$ -mediated EB-independent regulation. Some splice variants of ER $\beta$  can function as transcription factors in the absence of ligand resulting in increased expression of several proteins including CRH (Miller et al., 2004) and CRH binding protein (van de Stolpe et al., 2004). Thus it would follow that decreasing ER $\beta$  expression with the ER $\beta$ -siRNA-Ads could decrease the transcription of CRH and reduce plasma ACTH levels. On the other hand, in the presence of EB, the activation of CRH neurons is regulated by both ER $\alpha$  and ER $\beta$  with opposite effects (Weiser and Handa, 2009). EB-induced activation of ER $\alpha$  in GABAergic neurons of the peri-PVN region impairs glucocorticoid-dependent negative feedback regulation of the HPA axis, resulting in an increase in ACTH release (Weiser and Handa, 2009). In contrast, activation of ER $\beta$  in the PVN slightly suppresses ACTH release (Weiser and Handa, 2009). Thus, the net effect of EB on HPA axis activity depends on the balance of ER $\alpha$  and ER $\beta$  actions. In ER $\beta$ -siRNA-Ad treated rats, the activity of ER $\beta$  is decreased, resulting in an unopposed action of ER $\alpha$  in response to EB treatment that may explain why the ACTH response to 8-OH-DPAT is potentiated in these rats. Further studies are needed to test these possible underlying mechanisms.

Several groups have shown that estrogen regulates 5-HT $_{1A}$  receptors in various regions of the brain, but the specific estrogen receptor subtypes involved have not been directly addressed as in this current study. We focused on the regulation of 5-HT $_{1A}$  receptors in the PVN by estrogen because this region is important in the regulation of stress and the control of mood (Carrasco and Van de Kar, 2003; Legros et al., 1993; Raadsheer et al., 1995). Since desensitization of pre-synaptic and post-synaptic 5-HT $_{1A}$  receptors may be an underlying mechanism by which SSRIs slowly mediate their therapeutic effects (Bosker et al., 2001; Chaput et al., 1986; Czachura and Rasmussen, 2000; Kreiss and Lucki, 1995), it is important to determine which estrogen receptors are capable of rapid desensitization of 5-HT $_{1A}$  receptor function. The current study suggests that in the PVN the 5-HT $_{1A}$  receptor desensitization is independent of ER $\beta$ . It was previously demonstrated that 1) 5-HT $_{1A}$  receptors in the PVN mediate the increased release of oxytocin and ACTH that is induced by peripheral injections of 8-OH-DPAT (Osei-Owasu et al., 2005) and 2) that the

desensitization response induced by peripheral estradiol injections can be blocked by intra-PVN injections of pertussis toxin Xu et al., 2008). Further evidence for a direct effect of EB on desensitization of 5-HT<sub>1A</sub> receptors in the PVN comes from our previous study that demonstrated increased expression RGSz1 in the PVN with EB treatment (Carrasco et al., 2000, Neuroscience). An increase in RGSz1 can underlie the desensitization response since RGSz1 terminates Gz signaling and we have demonstrated that Gz is the Galpha protein that mediates 5-HT<sub>1A</sub> receptor stimulated release of oxytocin and ACTH (Serres et al., 2000, J Neuroscience). However, indirect effects of EB on 5-HT<sub>1A</sub> receptor signaling are possible as are direct effects of EB on oxytocin. Future studies would have to address whether or not ER $\beta$  regulates 5-HT<sub>1A</sub> receptors in other regions of the brain and also whether ER $\alpha$  is involved in 5-HT<sub>1A</sub> receptor regulation in the PVN and other brain regions. Determining which estrogen receptors mediate the EB-induced desensitization of 5-HT<sub>1A</sub> receptor signaling may allow us in the future to design treatments that activate specific estrogen receptor subtypes as an adjunct treatment with SSRIs for depression and other mood disorders.

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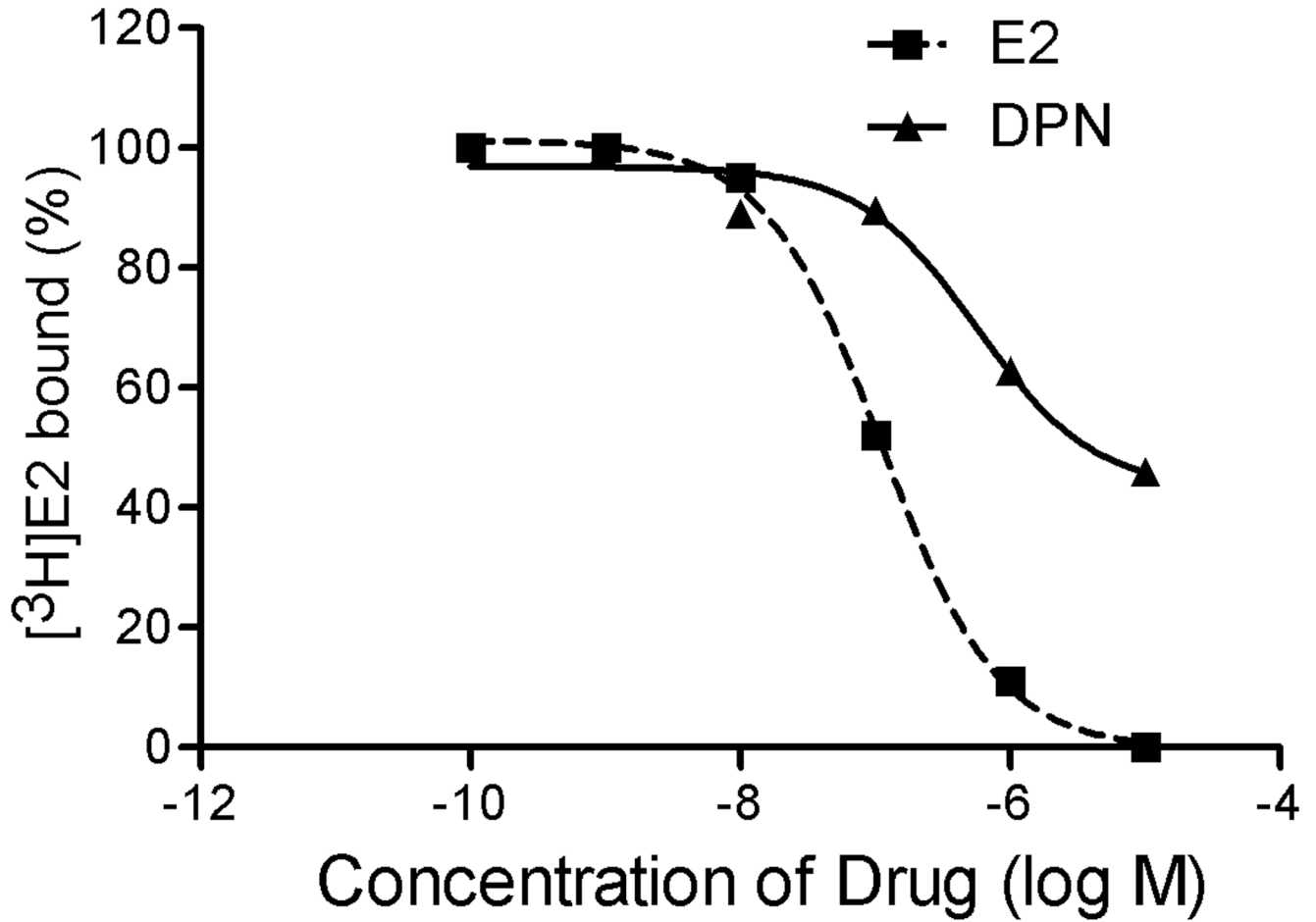
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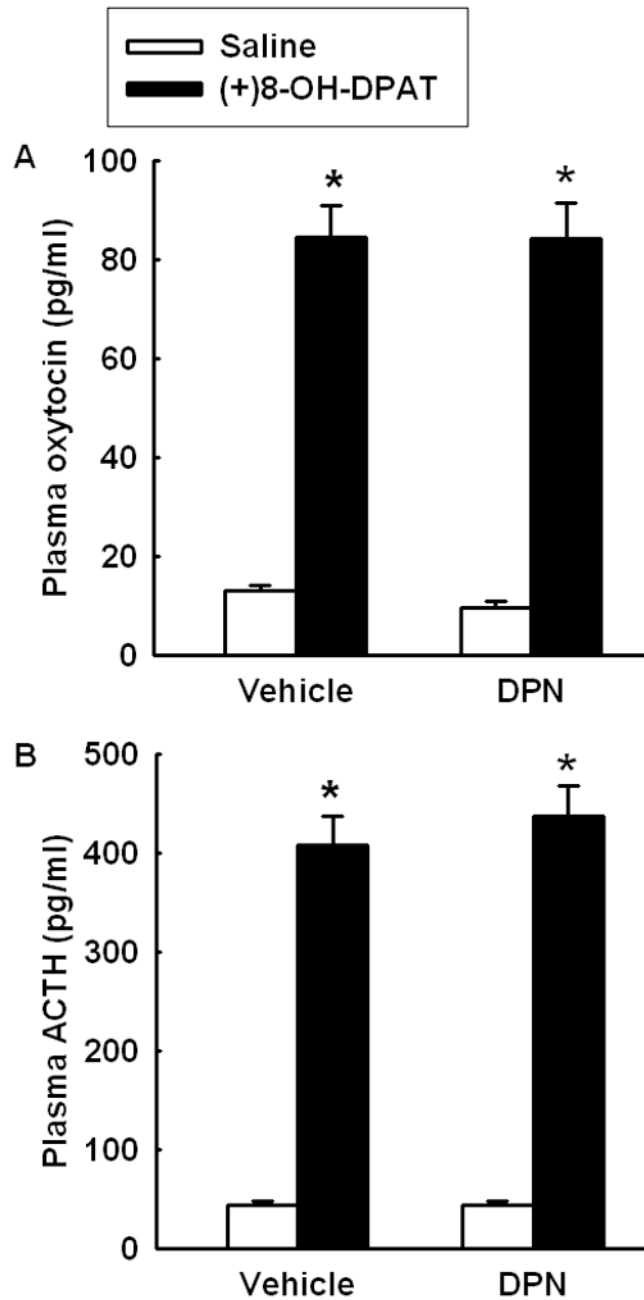
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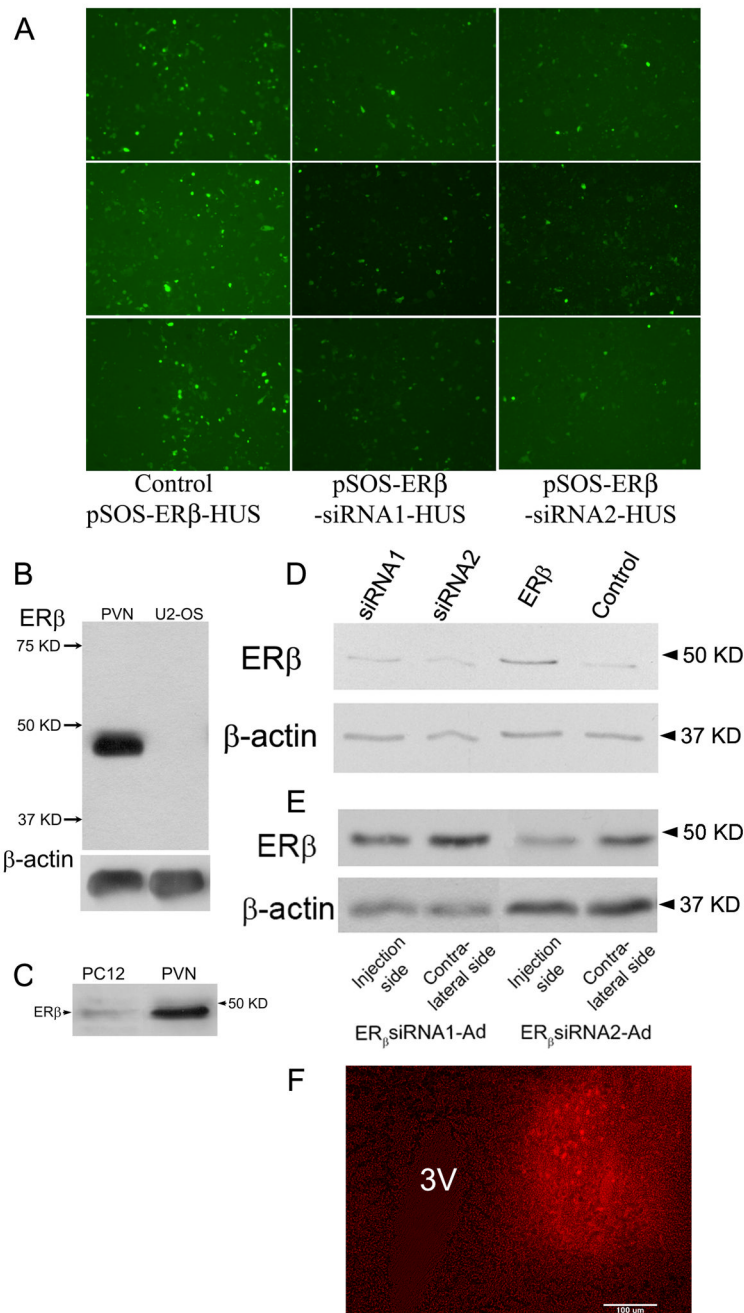


**Figure 1.** Example of competition curves of EB and DPN binding in plasma membranes of HEK293 cells stably transfected with GPR30. The data were expressed as a percentage of maximum specific  $[^3\text{H}]$ E2 binding.



**Figure 2.** Oxytocin and ACTH response to (+)8-OH-DPAT in DPN- or vehicle-treated animals. A. Plasma oxytocin and B. plasma ACTH levels expressed as the mean  $\pm$  SEM for 7–10 animals. \*Significant difference from saline injected animals,  $p < 0.05$  by Student-Newman-Keuls post hoc test.

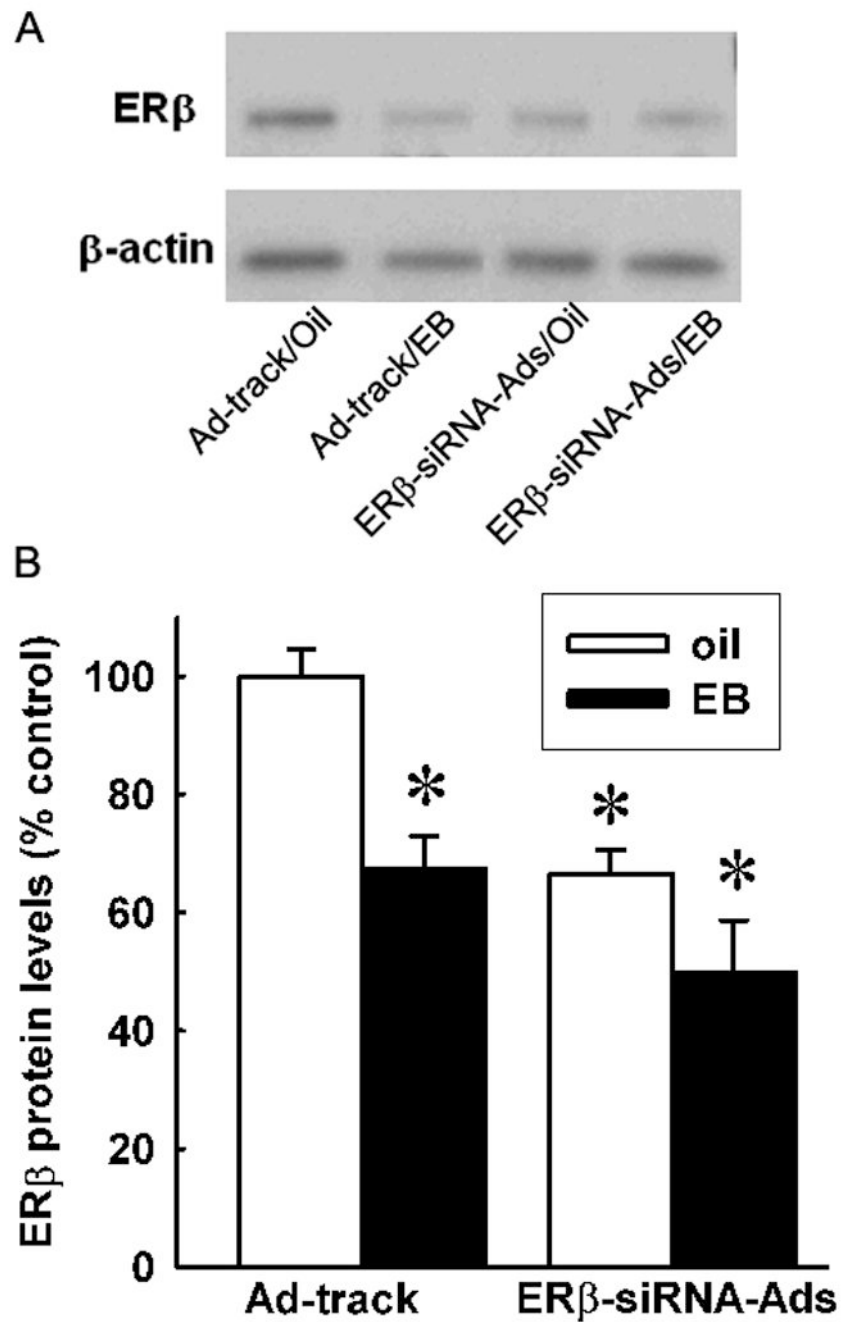




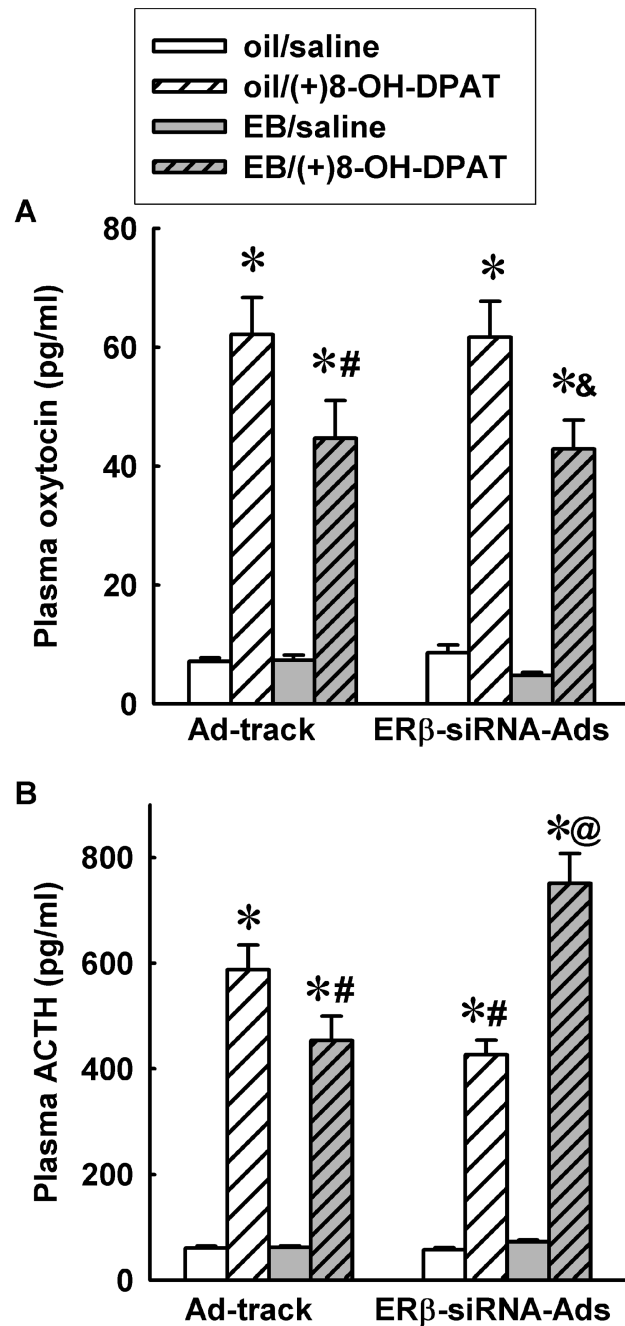
**Figure 3.**

Selection and evaluation of ER $\beta$  siRNAs. A. Selection of ER $\beta$  siRNA1 and 2 in SOS-HUS vector. SOS-ER $\beta$ -siRNA-HUSs or SOS-ER $\beta$ -HUSs were transfected into HEK 293 cells. The number and brightness of GFP-containing cells were compared between SOS-ER $\beta$ -siRNA-HUSs and SOS-ER $\beta$ -HUS transfected cells. Both vectors express GFP-tagged ER $\beta$  so a decrease in GFP intensity in the cells transfected with SOS-ER $\beta$ -siRNA-HUSs indicates a decrease in ER $\beta$  expression due to the siRNA. Each construct was transfected into three wells which are represented in each column. B. Identifying the selectivity of ER $\beta$  antibody: an immunoblot prepared with the ER $\beta$  antibody and homogenate from rat PVN (5  $\mu$ g protein) and a negative control, lysates from U-2 OS cells (5  $\mu$ g protein) which do not

express ER $\beta$ . The blot was then re probed with an actin antibody to demonstrate equal loading of protein in each lane. C. Immunoblot prepared with the ER $\beta$  antibody demonstrates that a single band at approximately 48 KD was detected in both the homogenate from rat PVN and PC12 cells lysates. D. Immunoblot for ER $\beta$  from PC12 cells transfected with different constructs. siRNA1, siRNA2 and ER $\beta$  represent the cells transfected with SOS-ER $\beta$ -siRNA1-HUS, SOS-ER $\beta$ -siRNA2-HUS and SOS-ER $\beta$ -HUS, respectively. The control designation indicates non-transfected cells. E. Example of an immunoblot for evaluation of the knockdown effect of ER $\beta$  with virally expressed siRNA *in vivo*. High titer ER $\beta$ -siRNA1-Ad and ER $\beta$ -siRNA2-Ad were unilaterally injected into the PVN of rats. ER $\beta$  levels in the injected side were compared with the contralateral side. F. An example of RFP labeling in the PVN to show the position of the viral injection. The images were captured from a fresh frozen brain section. The left panel was captured under fluorescent light, while the right panel was captured under bright field and fluorescent light combined in order to show the location of injection relative to the third ventricle (3V). Calibration bar indicates 100 microns.



**Figure 4.** Effect of ERβ- siRNA-Ads and EB injection on ERβ protein levels in the PVN. A. An example of an immunoblot of ERβ and β-actin. B. Western blot data analysis of ERβ. Data are expressed as mean ± SEM for 9–10 animals. \*Significant difference from control virus/oil injected animals,  $p < 0.05$  by Student-Newman-Keuls post hoc test.



**Figure 5.**

Effects of ERβ-siRNA-Ads on EB-induced alteration in the plasma oxytocin (A) and ACTH (B) response to (+)8-OH-DPAT. Data are presented as mean ± SEM (n=4–10). \*: Significant difference from saline-challenged animals with same pre-treatment., #: significant difference from control virus/oil/(+)8-OH-DPAT injected animal, &: significant difference from ERβ-siRNA-Ads/oil/(+)8-OH-DPAT injected animals, @: significant difference from ERβ-siRNA-Ads/oil/(+)8-OH-DPAT injected, control virus/oil/(+)8-OH-DPAT injected, and control virus/EB/(+)8-OH-DPAT injected animals, (p<0.05) by Student-Newman-Keuls post hoc test.