ESTROGEN IMPAIRS GLUCOCORTICOID DEPENDENT NEGATIVE FEEDBACK ON THE HYPOTHALAMIC–PITUITARY–ADRENAL AXIS VIA ESTROGEN RECEPTOR ALPHA WITHIN THE HYPOTHALAMUS

M. J. WEISER and **R. J. HANDA**[*]

Department of Biomedical Sciences, Neuroscience Division, Colorado State University, Fort Collins, CO 80523, USA

Abstract

Numerous studies have established a link between individuals with affective disorders and a dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis, most notably characterized by a reduced sensitivity to glucocorticoid negative (−) feedback. Furthermore there is a sex difference in the etiology of mood disorders with incidence in females being two to three times that of males, an association that may be a result of the influence of estradiol (E2) on HPA axis function. In these studies, we have examined the effect of E2 on glucocorticoid-mediated HPA axis (−) feedback during both the diurnal peak and the stress-induced rise in corticosterone (CORT). Young adult female Sprague–Dawley (SD) rats were ovariectomized (OVX) and 1 week later treated subcutaneous (s.c.) with oil or estradiol benzoate (EB) for 4 days. On the 4th day of treatment, animals were injected with a single dose of dexamethasone (DEX), or vehicle. EB treatment significantly increased the evening elevation in CORT and the stress-induced rise in CORT. In contrast, DEX treatment reduced the diurnal and stress induced rise in CORT and adrenocorticotropic hormone (ACTH), and this reduction was not apparent following co-treatment with EB. To determine a potential site of E2's action, female SD rats were OVX and 1 week later, wax pellets containing E2, the estrogen receptor beta (ERβ) agonist diarylpropionitrile (DPN), or the estrogen receptor alpha (ERα) agonist propylpyrazoletriol (PPT), was implanted bilaterally and dorsal to the paraventricular nucleus of the hypothalamus (PVN). Seven days later, animals were injected s.c. with a single dose of DEX, or vehicle to test for glucocorticoid-dependent (−) feedback. Results show that E2 and PPT increased, while DPN decreased the diurnal peak and stress-induced CORT and ACTH levels as compared to controls. Furthermore, E2 and PPT impaired the ability of DEX to inhibit both the diurnal and the stress-induced rise in CORT and ACTH, whereas DPN had no effect. Neuronal activation was measured by *c-fos* mRNA expression within the PVN following restraint. E2 and PPT increased *c-fos* mRNA, and impaired the normal DEX suppression of neuronal activation in the PVN. Taken together, these data indicate that estradiol causes a dysregulation of HPA axis (−) feedback as evidenced by the inability of DEX to

[*]Correspondence to: (current address) R. J. Handa, Department of Basic Medical Sciences, University of Arizona College of Medicine, Phoenix, 425 North 5th Street, Phoenix, AZ 85004, USA. Tel: 1-602-827-2161; fax: 1-602-827-2144. rhanda@arizona.edu (R. J. Handa).
Current address: R. J. Handa, Department of Basic Medical Sciences, University of Arizona College of Medicine, Phoenix, 425 North 5th Street, Phoenix, AZ 85004, USA.

suppress diurnal and stress-induced CORT and ACTH secretion. Additionally, the ability of E2 to inhibit glucocorticoid (−) feedback occurs specifically via ERα acting at the level of the PVN.

Keywords

stress; glucocorticoids; estrogen receptor; paraventricular nucleus; anxiety; depression

Major depressive disorder is a leading disability in the United States for young adults (Kessler et al., 2005). Women are twice as likely as men to experience a major depressive episode, and they tend to suffer a more severe form of depression and greater functional impairment (Angold and Worthman, 1993; Weissman et al., 1993; Thase et al., 1994; Kornstein, 1997). Other psychological disorders more prevalent in women include eating disorders and insomnia (Ehlert et al., 2001; Buckley and Schatzberg, 2005). Abnormalities in the hypothalamic–pituitary–adrenal (HPA) axis may underlie such pathologies as they are well documented in many mental disorders (Ehlert et al., 2001; Varghese and Brown, 2001; Barden, 2004). For example, the combined dexamethasone (DEX) suppression test (DST)/ corticotropin releasing hormone (CRH) challenge shows a sensitivity of up to 90% in detecting major depressive disorder (Varghese and Brown, 2001). Thus, the apparent dysregulation of the HPA axis in human affective disorders when put in context with a higher prevalence in women could indicate a role for estrogen in HPA axis dysfunction.

Hypothalamic-pituitary-adrenal (HPA) axis activation is a homeostatic mechanism that is triggered by a physical or psychological stressor (see; De Kloet et al., 2005, for review). Stress-related neuronal inputs are integrated at the level of the paraventricular nucleus (PVN) of the hypothalamus to induce the secretion of CRH and arginine vasopressin (AVP) into the hypophyseal portal vasculature. These neuropeptides stimulate the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary, which subsequently induces corticosteroid production by the adrenal cortex in humans (cortisol) and rodents (corticosterone; CORT). Importantly, HPA axis activation is terminated by a negative feedback action of glucocorticoids. Elevations in circulating CORT inhibit the HPA axis by acting via the glucocorticoid receptor (GR) in a direct and indirect fashion through the anterior pituitary, hypothalamus and hippocampus (Reul and De Kloet, 1985; Ratka et al., 1989). The main effect of elevated CORT is to decrease the synthesis and release of AVP, CRH and ACTH (Jingami et al., 1985).

In the brain, two distinct intracellular receptors have been shown to mediate the cellular responses to CORT (Reul and De Kloet, 1985). The type-I, or mineralocorticoid receptor (MR), is predominantly expressed in limbic structures such as the hippocampus (Reul et al., 1987). Type-II, or glucocorticoid receptor (GR), is expressed throughout the brain with highest amounts in hypothalamic areas such as the PVN and supraoptic nucleus (SON), as well as in the hippocampus. Both receptors are also responsible for mediating the negative feedback effects of CORT on neuroendocrine systems. MR exhibits a 10-fold higher affinity for CORT than GR, thus MR is predominantly occupied by basal CORT levels and is important in regulating basal secretion of CORT (Reul et al., 1987). The GR possesses a lower affinity for CORT and is thought to be the primary CORT receptor that modulates the

negative feedback response to elevated CORT levels, although MR is also involved in this (Ratka et al., 1989; Spencer et al., 1998; Pace and Spencer, 2005). Within the PVN, GR have been reported in both CRH and AVP containing neurons although there is much greater evidence for the presence of GR in AVP (Albeck et al., 1994) rather than in CRH neurons (Uht et al., 1988). Nonetheless, CORT acts to inhibit CRH and AVP expression in PVN neurons (Kovacs and Mezey, 1987; Swanson and Simmons, 1989), but in a differential manner (Ma and Aguilera, 1999; Kovacs et al., 2000). Whether glucocorticoid-mediated negative feedback on CRH and AVP neurons is truly direct or indirect is unknown.

Several studies have indicated that in the presence of estradiol there is an enhanced responsiveness of the HPA axis to a stressor which may be due in part to impairment of glucocorticoid negative feedback (Peiffer and Barden, 1987; Turner, 1990, 1992; Peiffer et al., 1991; Burgess and Handa, 1992, 1993; Carey et al., 1995; Ferrini et al., 1995; Patchev et al., 1995). Furthermore, gonad intact female rats have higher, whereas ovariectomized (OVX) females have similar, peak diurnal plasma CORT as compared to gonad intact males (Atkinson and Waddell, 1997; Seale et al., 2004). Potential mechanisms of action include competition between estrogen receptor alpha (ERα) and GR to regulate transcription at an AP-1 site (Uht et al., 1997), and reduction of GR binding and protein levels in the hippocampus, hypothalamus, and anterior pituitary (Burgess and Handa, 1992). In the preoptic area, a region with inhibitory PVN efferents, estradiol has been shown to alter glutamic acid decarboxylase (GAD)67 levels (Curran-Rauhut and Petersen, 2002). In addition to indirect effects on HPA axis, estrogen may directly influence activity within neurosecretory neurons in the PVN. This is suggested by studies showing a greater induction of c-fos mRNA within PVN neurons in response to stress when estradiol is present either systemically or directly administered to the PVN (Yukhananov and Handa, 1996; Rachman et al., 1998; Lund et al., 2004, 2006). Accordingly, studies have indicated that estradiol regulates several PVN neuropeptides (Levin and Sawchenko, 1993), and upregulates restraint-induced CRH mRNA (Lund et al., 2004).

In brain, the actions of estradiol can be mediated by two distinct estrogen receptors, ERα and ERβ. Importantly, this laboratory has recently shown that the HPA enhancing effect of systemic estradiol administration can be mimicked by the local application of 17β-estradiol or a selective ERα agonist to the PVN (Lund et al., 2006). ERα is not expressed within the PVN, yet is expressed in the peri-PVN, a region implicated in HPA axis inhibition (Suzuki and Handa, 2005). Within the PVN, ERβ is found predominantly in pre-autonomic neurons and thus is an unlikely site of a direct estradiol enhancement of HPA activity (Stern and Zhang, 2003). Taken together, these studies suggest that estradiol might impair the glucocorticoid-mediated negative feedback system directly or indirectly to modulate HPA axis activity. However, whether or not estradiol can alter glucocorticoid negative feedback has not been explicitly described.

In the studies described here, we have examined the possibility that estradiol impairs the sensitivity of the HPA axis to glucocorticoid negative feedback, by examining its effect on both the diurnal peak in CORT secretion and following a psychological stressor (restraint). Indeed, we have found that subtype-selective ER agonists when administered into the systemic circulation or locally to the peri-PVN region of the hypothalamus can impair

glucocorticoid negative feedback. These data indicate that in the presence of estradiol there is a dysregulation of the HPA axis that is demonstrated by the inability of DEX to suppress diurnal and stress-induced CORT secretion. This impairment in negative feedback occurs specifically via ERα acting at the level of the PVN.

EXPERIMENTAL PROCEDURES

Animals

Adult female Sprague–Dawley (SD) rats (~250 g) were obtained from Charles River Laboratories (Wilmington, MA, USA). The rats were caged in pairs, housed in the Colorado State University vivarium and maintained on a 12-h light/dark cycle (lights on at 07:00 h) with *ad libitum* access to rat chow and water. One week following arrival, rats were OVX under isoflurane anesthesia. All animal protocols were previously approved by the institutional animal care and use committee (IACUC) at Colorado State University. All procedures conformed to the Public Health Service policy on humane care and use of laboratory animals. Experiments were designed to minimize suffering and number of animals used.

Systemic hormone administration: diurnal experiment

One week following ovariectomy, animals were treated with oil or estradiol benzoate (EB, Sigma, St. Louis, MO, USA, 25 μg/kg; s.c.) daily for 4 days. On the fourth day of treatment, animals were injected s.c. with a single dose of DEX (Sigma) (30 μg/kg, s.c.) at 12:00 h. Controls received oil vehicle. Animals were sacrificed at 18:00 h (lights out at 19:00 h) on the same day; a time that is close to the diurnal peak in plasma CORT in our animal colony. Trunk blood was collected for CORT and ACTH analysis via radioimmunoassay (RIA).

Systemic hormone administration: stress experiment

One week following ovariectomy animals were treated with oil or EB (25 μg/kg, s.c.) daily for 4 days. On the fourth day of treatment, animals were injected s.c. with a single dose of DEX (30 μg/kg, s.c.) at 04:00 h 6 h prior to restraint stress. Restraint was performed by removing the animal from its home cage and placing it into a Plexiglas restraint tube (Plas-Laboratories, Lansing, MI, USA). Animals were sacrificed directly from the tube 20 min later. Controls received oil vehicle. Control animals for restraint were killed immediately after removal from their home cage. Animals were sacrificed after 20 min of restraint between 10:00 and 11:00 h and trunk blood samples collected for CORT and ACTH analysis via RIA.

Stereotaxic implantation

One week following ovariectomy animals were fitted bilaterally with two 22 gauge stainless-steel cannulae (Small Parts, Miami Lakes, FL, USA) with the aid of a small animal stereotaxic instrument (David Kopf Instruments, Tujunga, CA, USA). The tips of the cannulae were previously packed with one of the following compounds: EB, propylpyrazoletriol (PPT) (ERα agonist; relative binding affinity (RBA)=49; Stauffer et al., 2000; Tocris, Ellisville, MO, USA), or diarylpropionitrile (DPN) (estrogen receptor beta agonist; RBA=18; Meyers et al., 2001; Tocris), dissolved into warmed beeswax to a final

concentration of 0.5 μM, and packed to a height of 0.5 mm within the end of the cannulas. Controls received cannulae packed with wax alone. Stereotaxic coordinates to allow placement of the cannula tip to the region just above the PVN were lateral 10° insertion angle to 1.8 mm posterior and 2.0 mm lateral to bregma, and 6.5 mm below the skull surface. A 28 gauge stainless steel wire cut to extend 1.0 mm past the length of the cannulas was inserted into the cannulae and the pellet expelled. Following sacrifice of all animals, confirmation of pellet placement was confirmed in Cresyl Violet–stained brain sections. Animals where both pellets were >0.5 mm away from the PVN were excluded based on previous studies using the same pellet hormone concentration and resulting spread (~0.5 mm for estradiol) (Lund et al., 2005).

Local hormone administration: diurnal experiment

Seven days after stereotaxic surgery, animals were injected s.c. with a single dose of DEX (Sigma) (30 μg/kg, s.c.) at 12:00 h. Controls received oil vehicle. Animals were sacrificed directly from the home cage at 18:00 h (lights out at 19:00 h) the same day, near the diurnal peak in plasma CORT, and trunk blood was collected for CORT analysis using RIA.

Local hormone administration: stress experiment

Seven days after stereotaxic surgery, animals were injected s.c. (04:00 h) with a single dose of DEX (30 μg/kg) 6 h prior to restraint stress (at 10:00 h). Controls received oil vehicle. Animals were sacrificed after 20 min of restraint at 10:20 h and trunk blood samples collected for CORT and ACTH analysis via RIA.

CORT RIA

Trunk blood was centrifuged at 4 °C and plasma removed and stored at −20 °C until assayed. For assay, plasma samples were diluted in 0.01 M PBS (1:25) and corticosteroid binding globulin (CBG) was inactivated by incubation at 65 °C for 1 h. Samples (20 μl) and standards (5–700 ng/ml) were incubated overnight at 4 °C with antiserum (rabbit anti-CORT; MP Biomedicals, Solon, OH, USA) and [3H] CORT (PerkinElmer, Boston, MA, USA) in 0.1% gelatin 0.01 M PBS. Free CORT was separated from antibody-bound CORT with 1.0 ml dextran-coated charcoal. After centrifugation, the supernatant containing antibody-bound CORT was mixed with 4 ml of scintillation fluid and counted with a Packard 2900 TR liquid scintillation counter (Meriden, CT, USA). The intra-assay and inter-assay variance as measured by internal quality controls was 4.5% and 7.8% respectively.

ACTH RIA

For assay, plasma samples were diluted in PBS-albumin (0.01 M PBS, 0.9% NaCl, 0.1% albumin, 100,000 kIUs aprotinin/l). Samples and standards (5–2000 pg per tube) were incubated overnight at 4 °C with antiserum (rabbit anti-ACTH) (Immunostar, Hudson, WI, USA) and 2% normal rabbit serum. The following day, [125I] ACTH (1–39) (Amersham, Piscataway, NJ, USA) in PBS-albumin (100 μl) was added to each tube as the tracer, and incubated overnight at 4 °C. On the third day, goat anti-rabbit gamma globulin (Calbiochem, La Jolla, CA, USA) was added to all tubes except totals, and tubes incubated overnight at 4 °C. On the last day, 3 ml of PBS-albumin was added and tubes were centrifuged

(>1000×*g*). Assay tubes were immediately decanted, blotted dry, and the resulting pellet counted with a Packard cobra II gamma counter. The intra-assay and inter-assay variance as measured by internal quality controls was 3.8% and 6.1% respectively.

In situ hybridization

In situ hybridization for *c-fos* mRNA was performed as previously described (Lund et al., 2004) utilizing a 48-bp oligonucleotide probe targeted to the rat *c-fos* gene. The oligonucleotide probe was labeled using ^{35}S-dATP. Following hybridization slides were apposed to autoradiographic film (Kodak BioMax MR, Eastman Kodak, Co., Rochester, NY, USA) and allowed to expose for 10 days. Autoradiograms were analyzed for density using a video camera (Sony XC-77, Tokyo, Japan) attached to a Nikon lens (Melville, NY, USA). Scion Image (Frederick, MD, USA) was utilized to count density per a fixed region encompassing the PVN. For each section, background density was obtained from a region adjacent to the PVN, and subtracted from the PVN measurement. Bilateral measurements were obtained from four separate sections and averaged to obtain the value for each individual animal.

Immunocytochemistry

To examine co-localization of ER*α* and GAD67, four OVX'd young adult (300 g) female SD rats were intracardially perfused with 4% buffered paraformaldehyde. Brains were postfixed in 4% paraformaldehyde, cryoprotected in 30% sucrose, sectioned at 35 μM on a Leitz cryostat, and stored in cryopreservative at −20 °C until further processed for immunohistochemical detection of ER*α* and GAD67. After standard washes, the free floating sections were incubated in 6% normal goat serum (NGS) in 0.1 M PBS with 0.1% triton-x (TX) to block nonspecific binding, then incubated for 72 h at 4 °C with the ER*α* antibody (C1355; detects ligand-bound and unbound ERα; obtained from Dr. M. Shupnik, UVA; 1:10,000 dilution) in 0.1 M PBS with TX in the presence of 2% NGS. Next, the tissue was washed three times for 10 min each in PBS with TX and incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA; 1:500) in PBS with TX in the presence of 2% NGS for 2 h at room temperature. Sections are subsequently washed and processed according to the avidin–biotin–peroxidase procedure (Vector Laboratories; 1:500). After standard washes, the tissue was rinsed in 0.1 M Tris-buffered saline (pH 8.0) for 15 min and then developed with 3,3′-diaminobenzidine (DAB; 0.5 mg/ml; (Sigma) in 0.1 M Tris-buffered saline) containing 0.2% nickel sulfate and 0.01% hydrogen peroxide for 3–5 min to produce a blue–black reaction product. The reaction is stopped by several washes in 0.1 M PBS. Subsequently, the sections were incubated for 48 h at 4 °C with the mouse GAD67 antibody (Chemicon, Temecula, CA, USA; 1:10,000 dilution) in PBS containing 0.01% TX and 2% NGS. The sections were then washed and processed according to the avidin–biotin–peroxidase procedure (Vector Laboratories; 1:500). After standard washes, the tissue is rinsed in 0.1 M Tris-buffered saline (pH 8.0) for 15 min and then developed with DAB (0.5 mg/ml (Sigma) in 0.1 M Tris-buffered saline) containing 0.01% hydrogen peroxide for 3–5 min to produce a brown reaction product. Control sections, where primary antibody was omitted, were run in parallel. Complete loss of staining for the corresponding antigen was seen in these sections.

Data analysis

Analysis of variance was performed on data from each experiment using the StatView data analysis software (Abacus Concepts, Inc., Berkeley, CA, USA). Fisher's protected least squares difference (PLSD) analysis was used post hoc where appropriate. Differences were considered significant when $P<0.05$. Data are expressed as group means±SEM.

RESULTS

Estradiol treatment augments the diurnal rise of CORT and ACTH and prevents DEX suppression of CORT and ACTH

Females have a more robust diurnal CORT rhythm as compared to males, perhaps in part due to altered glucocorticoid negative feedback (Atkinson and Waddell, 1997). Therefore, this experiment was designed to determine the effect of estradiol on glucocorticoid-mediated negative feedback during the diurnal rise in CORT secretion. EB alone significantly ($P<0.01$) increased plasma CORT and ACTH levels (Fig. 1). DEX treatment also significantly ($P<0.01$) suppressed serum CORT levels in vehicle-treated animals. In contrast, in EB-treated animals, CORT and ACTH levels were not suppressed by DEX. Thus, in the presence of estradiol, the synthetic glucocorticoid DEX is unable to suppress the diurnal rise in CORT secretion.

Estradiol treatment augments restraint-induced CORT and ACTH secretion and prevents DEX suppression of restraint-induced CORT and ACTH

Females also have a more robust hormonal response to a stressor as compared to males, possibly driven by gonadal hormone influences on glucocorticoid negative feedback (Burgess and Handa, 1992; Handa et al., 1994). Therefore, this experiment was designed to determine the effect of estradiol on GR-mediated negative feedback on stress-induced activation of the HPA axis. Estrogen alone significantly ($P<0.01$) increased stress-induced CORT and ACTH levels (Fig. 2). EB+DEX animals showed significantly ($P<0.01$) higher plasma CORT and ACTH compared to vehicle+DEX animals. In oil-treated animals, DEX treatment suppressed CORT by an average of 289.6 ng/ml or 62.9% and ACTH by an average of 365.7 pg/ml or 74.9%. In contrast, when pretreated with EB, the DEX-treated animals showed a 199.8 ng/ml or 34.4% on average suppression of CORT and a 281.9 pg/ml or 56.7% suppression of ACTH. Overall, CORT and ACTH values for EB+DEX animals were similar to those of oil+vehicle controls. Thus, in the presence of estradiol, the stress-induced levels of CORT and ACTH are significantly increased and responsiveness to DEX is significantly decreased.

Estrogen acts via ERα near the PVN to augment the diurnal rise of CORT and ACTH and to prevent DEX suppression of CORT and ACTH

Given the matching ACTH and CORT data from the previous two experiments, estradiol likely acts at the level of the brain or pituitary to impair glucocorticoid negative feedback. Therefore, this experiment was designed to determine a potential site for estradiol's effect on glucocorticoid negative feedback during the diurnal rise in CORT, and also to determine whether this effect is through an ERα-or ERβ-dependent mechanism. Animals were

stereotaxically implanted with wax pellets containing ER agonists targeted to an area just superior to the PVN (Fig. 3). Estradiol and PPT significantly increased ($P<0.01$), whereas DPN significantly lowered ($P<0.01$), CORT and ACTH when compared to controls given a wax-only pellet (Fig. 4). DEX treatment significantly ($P<0.01$) lowered CORT and ACTH levels in control and DPN-implanted animals, however estradiol and PPT significantly ($P<0.01$) impaired the suppression by DEX.

Estrogen acts via ERα near the PVN to augment restraint-induced CORT and ACTH secretion and to impair DEX suppression of restraint-induced CORT and ACTH

Results from the previous experiment indicated that estradiol may act via ERα at sites near the PVN to impair glucocorticoid dependent HPA negative feedback during the diurnal rise in CORT. We postulated that this may be also found following restraint stress activation of the axis. As in the previous experiment, animals were treated with hormone by stereotaxic implantation of wax pellets containing ER agonists that were targeted to a site just superior to the PVN. Twenty minutes after the start of restraint, estradiol- and PPT-implanted animals had significantly higher ($P<0.01$) CORT and ACTH than did controls (Fig. 5). DPN-implanted animals had significantly lower ($P<0.01$) CORT and ACTH following restraint than did controls. Moreover, DEX treatment significantly lowered ($P<0.01$) CORT and ACTH in both controls and DPN-implanted animals, however estradiol and PPT treatment significantly impaired ($P<0.01$) the ability of DEX to inhibit CORT and ACTH secretion.

Estrogen acts via ERα near the PVN to augment restraint-induced PVN *c-fos* mRNA expression and to impair DEX suppression of restraint-induced PVN *c-fos* mRNA expression

During a stressor, neural activity in the PVN is significantly increased, and can be subsequently suppressed with glucocorticoids. It follows then, that estradiol should impair glucocorticoid suppression of neural activity in the PVN. In order to couple our hormonal data with corresponding examination of neuronal activation following the restraint stress, we measured *c-fos* mRNA expression in the PVN. *c-fos* mRNA expression was significantly ($P<0.05$) higher in estradiol- and PPT-implanted animals and lower in DPN-implanted animals as compared to controls (Fig. 6). DEX treatment significantly reduced *c-fos* mRNA expression following restraint in controls and DPN treated animals, however estradiol and PPT treatment significantly impaired ($P<0.01$) the ability of DEX to inhibit *c-fos* mRNA expression.

ERα is expressed in GABAergic neurons surrounding the PVN

To determine the phenotypes of ERα-containing neurons in and around the PVN, we examined ERα immunoreactivity (IR) in OVX young adult female SD rats given replacement estradiol. Nuclear ERα-IR was sparse within the PVN and periventricular PVN, but ERα-IR was seen surrounding the PVN proper especially in the dorsal region (Fig. 7). Moreover, dual-immunocytochemistry revealed that ERα containing neurons in the peri-PVN also contained GAD (67.4%±6.1% of ER positive cells contained GAD67). Thus, ERα is expressed in the PVN surround, and is predominantly found in GABAergic neurons.

DISCUSSION

In healthy individuals the physiological product of the stress axis, corticosteroids (cortisol in humans, CORT in rodents), normalize the activity of the axis via negative feedback at multiple sites including the hypothalamus, pituitary gland, and adrenal cortex. Impairment of tonic or phasic HPA axis inhibition results in abnormal endogenous glucocorticoid secretory patterns and levels, which are correlated with several disease states such as hypertension, inflammation, obesity, heart disease and diabetes (Baid and Nieman, 2004; Whitworth et al., 2005; Pasquali et al., 2006; Tomlinson and Stewart, 2007; Tait et al., 2008).

Gonadal steroid hormones play a vital role in modulating HPA axis function. It is well established that basal and stress-induced adrenal steroid secretion is greater in female rodents than in males (Critchlow et al., 1963; Kitay, 1963; Handa et al., 1994), and that the activational effects of gonadal steroids play an integral role in this sex difference (Sencar-Cupovic and Milkovic, 1976). In females, ovariectomy reduces stress-induced CORT and ACTH, and this is reversed by estrogen treatment (Burgess and Handa, 1992; Handa et al., 1994; Suzuki et al., 2001). However, this is not always the case as several groups have reported that estrogen can inhibit responses to stress (Young et al., 2001; Figueiredo et al., 2002; Ochedalski et al., 2007). Furthermore, human studies indicate than men typically respond stronger hormonally to a stressor than women [for review see; Kudielka and Kirschbaum, 2005], however a short-course treatment of estradiol in young men significantly increases cortisol levels following a social stress test (Kirschbaum et al., 1996). Interestingly, women also have higher weekday wakening salivary cortisol levels following an examination stress as compared to men (Weekes et al., 2008). These discrepancies may be due in part to opposing actions of estradiol signaling through ERα and ERβ. However, at present the mechanism whereby estrogen alters HPA axis function is unknown.

In the studies described here, we investigated the effect of estradiol on glucocorticoid-mediated negative feedback. Basal HPA axis activity is circadian in nature, where a diurnal peak in secretion occurs just prior to onset of activity (early morning for humans, and early evening for rodents). Therefore, we examined basal glucocorticoid negative feedback at the diurnal peak in CORT in addition to stress-activated CORT secretion. Furthermore, we determined which estrogen receptor subtype is responsible for this effect and the brain site mediating estradiol's effects on glucocorticoid-mediated negative feedback. Based on our results, we believe that estradiol exerts its action at the level of the PVN, through an ERα-mediated disruption of GR-mediated negative feedback.

Glucocorticoid-mediated HPA axis negative feedback is dependent upon the dose and the duration of exposure (Abe and Critchlow, 1980). Normal function of the HPA axis is dependent upon permissive and reactive actions of glucocorticoids (for review see; Sapolsky et al., 2000). For example, adrenalectomy (ADX) results in higher PVN neuropeptide synthesis and secretion both at the basal and stressed states. Additionally, feedback can occur either directly at the level of the CRH and AVP neurons of the PVN as well as the corticotrophs of the anterior pituitary, or indirectly though GR and MR containing brain regions that project to the PVN such as the hippocampus and amygdala (Davidson and Feldman, 1967; Bohus and Strashimirov, 1970; Dallman et al., 1987; Sawchenko, 1987).

Glucocorticoid effects vary according to duration of exposure. Fast feedback occurs within seconds to minutes, whereas delayed feedback occurs within minutes to hours (Dallman et al., 1987). Fast feedback occurs in a time frame that is too short for de novo protein synthesis, and must be attributable to non-genomic actions of glucocorticoids. These actions have been shown to involve the classical GR and MR as well as endocannabinoids (Widmaier and Dallman, 1984; Hinz and Hirschelmann, 2000; Di et al., 2003, 2005; Patel et al., 2004). Delayed feedback occurs in a time frame that likely involves influences on gene transcription and de novo protein synthesis and is attributable to activation of GR and/or MR, and may be direct or indirect in nature. For the purpose of this study we examined delayed glucocorticoid-mediated HPA axis negative feedback.

In order to more closely relate our findings to the function of the HPA axis in the human condition of depression, we utilized a DST. The DST is a well-established clinical predictor of depression (Carroll and Curtis, 1976), and has been used in rodents as a model for the human test (Lurie et al., 1989). More recently, the combined DEX/CRH test, where DEX suppression in the presence of HPA axis drive (CRH), has been proven to be 90% accurate in diagnosing depressed individuals (Varghese and Brown, 2001). Furthermore, the DEX/CRH test has been used to accurately predict relapse and can be used as a biomarker to positively identify novel treatments for depression (Aubry et al., 2007; Ising et al., 2007). The DST and DEX/CRH test measure common pathology, however the DEX/CRH has better diagnostic reliability (Watson et al., 2006). Therefore, in this study, we examined the DST under the basal state as well as under HPA axis drive (restraint stress) to better match the parameters of the DEX/CRH test utilized in humans.

In our initial set of experiments, we found that the synthetic glucocorticoid DEX, suppressed peak diurnal and restraint-induced CORT in OVX females. The degree of suppression was slightly higher following restraint than during the diurnal peak. This may be in part due to the higher overall activity of the HPA axis following a stressor, or due to decreased glucocorticoid sensitivity during diurnal peak secretion. This decreased sensitivity is thought to be partly responsible for the normal diurnal elevation in CORT (Bradbury et al., 1994). Interestingly, following estradiol replacement to OVX females, the diurnal increase in CORT was significantly increased and DEX suppression of the diurnal peak was completely inhibited. Therefore, estradiol acts to inhibit glucocorticoid negative feedback during the diurnal rise in CORT. Moreover, estradiol treatment increased CORT following a restraint stress and impaired DEX suppression of stress-induced CORT. In both cases, the impairment was not due to estradiol's actions at the adrenal cortex, as the pattern of plasma CORT closely matched plasma ACTH in all animals. This provides strong evidence that estradiol directly impairs glucocorticoid-mediated negative feedback. These results also suggest that the increased responsiveness to a stressor that is seen in females is likely due to decreased sensitivity to glucocorticoid feedback.

Results from these experiments indicated that the impairment in negative feedback must occur either in the brain or at the level of the pituitary gland. Previous data from this laboratory indicated that estradiol placed centrally near the PVN of the hypothalamus of OVX female rats resulted in a stronger hormonal response to a restraint stress than controls (Lund et al., 2006). Additionally, in a separate earlier study, placement of DEX near the

PVN blunted CRH hnRNA transcription following a stressor (Kovacs et al., 1986; Sawchenko, 1987). Therefore, we designed studies to determine the responsible estrogen receptor subtype and the location of estradiol's effect on glucocorticoid negative feedback. Our data implicate the peri-PVN region as a critical brain site integrating estradiol influence on HPA axis output and glucocorticoid-mediated negative feedback. Our studies utilized the same procedure used previously where wax pellets containing subtype selective agonists PPT (ERα) and DPN (ERβ) in addition to the endogenous ER ligand estradiol were placed bilaterally to a region just above the PVN. This provides for continuous local release confined to a 0.5 mm radius around the wax pellet that allows for the examination of local ER activation independent of peripheral effects (Lund et al., 2006).

We found that activation of ERα in the region of the PVN increased the diurnal gain of the HPA axis and was sufficient to block all suppressive effects of DEX during the diurnal peak in CORT. Following a restraint stress, peri-PVN ERα activation significantly impaired DEX suppression and resulted in CORT and ACTH levels similar to untreated, stressed controls. As expected, *c-fos* mRNA expression following the restraint stress closely matched the hormonal output of the axis. Animals implanted with estradiol or PPT pellets had stronger activation of PVN neurons following a stressor than control or DPN-implanted animals, and this activation was not diminished by DEX to the same degree as seen in control animals. From these findings we conclude that estradiol impairs glucocorticoid-mediated HPA axis negative feedback during the diurnal peak and following a restraint stress specifically through activation of ERα-containing neurons in the region of the PVN.

The actions of estradiol can be mediated by both ERα and ERβ. Whereas ERβ is found in PVN neurons, ERα is not expressed in CRH or AVP neurons in the PVN (Suzuki and Handa, 2005), thus ruling out a direct action of estradiol through ERα on the function of these neurons. However, ERα is found in stress-responsive areas that provide inhibitory GABA input into the PVN including the bed nucleus of the stria terminalis (BnST) and medial preoptic area (MPOA) (Shughrue et al., 1997) and the peri-PVN region (Herman et al., 2002). In support of a GABAergic mechanism, our studies show co-localization of ERα-IR with GAD67-IR. GAD67 is the rate-limiting enzyme in the production of GABA. Although we found that ERα-IR was largely absent from the PVN, it was present in neurons immediately surrounding the PVN (peri-PVN), especially dorsally. Additionally, a majority of these ERα-IR neurons in the dorsal peri-PVN region also contained GAD67-IR indicating that they are inhibitory interneurons. This region of the peri-PVN contains GAD-IR neurons that project to the parvocellular PVN (Roland and Sawchenko, 1993). Application of a GABA agonist locally to this region can enhance the CORT response to a stressor (Cullinan, 1998), presumably by inhibiting GABAergic transmission to hypophysiotropic CRH neurons. Therefore, it is plausible that estradiol may work via ERα to inhibit GABAergic transmission from these peri-PVN neurons.

Our findings are consistent with several other estrogen-sensitive systems that indicate a role for estrogen modulation of GABAergic neurotransmission. These include the actions of estrogens on dendritic spine growth (Murphy et al., 1998) and the positive-feedback response of gonadotropin-releasing hormone neurons (Herbison, 2008). Furthermore, estradiol has been shown to alter GAD67 expression in the AVPV and MPN (Curran-Rauhut

and Petersen, 2002). Estradiol-GABA interactions are also critical for proper development of the brain [for review see; McCarthy, 2008]. Given that the direct administration of estradiol or an ERα agonist to the PVN results in impaired glucocorticoid-mediated negative feedback, and the presence of ERα in GAD67 containing neurons surrounding the PVN, these results suggest that ERα might inhibit glucocorticoid negative feedback by reducing an inhibitory GABAergic input into the PVN.

Stress-integration at the level of the PVN involves both inhibitory GABA and excitatory glutamate circuits (see; Herman et al., 2002, 2004; Kovacs et al., 2004, for review). Recent studies have suggested that a substantial part of PVN excitation and inhibition is gated by local circuit neurons in the vicinity of the PVN. Studies have shown that microinjection of glutamate elicits, while local application of a glutamate receptor antagonist inhibits ACTH and CORT secretion (Darlington et al., 1989; Ziegler and Herman, 2000). Inhibitory GABA containing neurons synapse on parvicellular PVN neurons and are present in the immediate surround of the PVN, BnST, MPOA, lateral hypothalamic area (LHA), and anterior hypothalamic area (AHA) (Boudaba et al., 1996; Miklos and Kovacs, 2002). These regions also provide stress responsive afferents surrounding the PVN (Cullinan et al., 1996; Bali et al., 2005). In addition, microinjection of muscimol (GABA-A receptor agonist) into the PVN attenuates, while bicuculline (GABA-A receptor antagonist) augments stress-induced ACTH secretion (Dimicco et al., 1996; Cole and Sawchenko, 2002). Furthermore, studies have shown stress-induced increases in the GABA-synthesizing enzyme GAD67 mRNA in PVN-projecting areas including peri-PVN, MPOA, BnST, arcuate nucleus, and hippocampus (Bowers et al., 1998). The presence of both ionotropic glutamate receptors and GABA-A receptors in CRH and AVP neurons indicates their ability to integrate inhibitory and excitatory inputs (Aubry et al., 1996; Cullinan et al., 1996). This is corroborated by studies showing local GABA-A receptor blockade increases CRH and AVP expression (Bali and Kovacs, 2003). Recent studies by (Di et al., 2005) have shown glucocorticoids secreted in response to a stressor act in a rapid feedback fashion to decrease glutamate and increase GABA release onto PVN neurons. Taken together, these studies suggest that local GABA circuits are key to stress integration at the PVN and play a vital role in glucocorticoid dependent negative feedback although other mechanisms of action for estrogen interference of glucocorticoid-mediated negative feedback must also be considered.

It is possible that our results reflect estradiol's ability to impair either GR or MR autoregulation and expression (Burgess and Handa, 1992; Patchev and Almeida, 1996), and thus impair glucocorticoid-mediated feedback. Earlier molecular studies have shown that GR and ER can interact in control of a reporter gene regulated through simple hormone response elements (Uht et al., 1997). How this relates to neuronal function is not entirely clear since studies have not definitively colocalized ERα or ERβ with GR or MR in brain regions known to be important in CORT-mediated negative feedback, and ERα has been shown to be absent in GR containing CRH and AVP neurosecretory neurons of the PVN. Until future studies indicate the existence of ERα and GR within the same cell population, it must be postulated that estradiol works indirectly to influence glucocorticoid negative feedback.

Although the effects of estradiol observed here may also be peripheral, causing inhibition of negative feedback at the level of the pituitary or adrenal glands, our results seem to rule out this possibility. The changes in CORT secretion that we observed were mimicked by ACTH secretion, and the local administration of hormone to the peri-PVN region rules out potential actions at peripheral sites. Alternatively, effects could involve alteration of CBG levels or binding capacity. Increased CBG levels might lead to decreased sensitivity to endogenous and exogenous corticosteroids, and thus lower glucocorticoid-mediated negative feedback. Circulating CBG levels are 2.5 times higher in females than in males, however this sex difference appears to be due to the organizational actions of sex hormones (Mataradze et al., 1992). Furthermore, estradiol treatment does not influence CBG levels in females (Mataradze et al., 1992; McCormick et al., 2002), and has been shown to increase or have no effect on CBG levels in males (McCormick et al., 2002; Lund et al., 2004). To circumvent this potential confound, we utilized a synthetic glucocorticoid (DEX), which does not bind CBG (Kolanowski and Pizarro, 1969; Koblinsky et al., 1972). DEX has been shown to inhibit HPA axis activity when administered directly to the PVN (Kovacs and Makara, 1988), and an intraperitoneal injection of femtomolar ^{3}H-DEX produces binding in glucocorticoid-concentrating brain regions within 1 h (Hassan et al., 1999).

An unlikely alternative possibility is that hormone or agonist could have diffused far enough from the pellet to enter the third ventricle, and thus enter cerebrospinal fluid (CSF) circulation. In this case, a di- or tri-synaptic mode of inhibition may be plausible, where estrogen could induce changes in GABA synthesis or release, in areas with indirect connections with the PVN like the hippocampus, lateral septum, or amygdala. We do not think that this is a viable alternative since the diffusion of hormone would result in CSF concentrations too low to appreciably effect ER signaling in distant brain regions.

Yet another possibility is that estradiol could increase GABA receptor levels in brain regions that provide inhibitory input to the PVN, thus increasing potential inhibition of these inhibitory PVN inputs. Indeed, some studies have shown that estrogen can increase $GABA_A$ receptor subunit expression in the MPA and BnST (Herbison and Fenelon, 1995). Whether these changes can affect glucocorticoid-mediated HPA negative feedback is uncertain. Such a mechanism is unlikely except at the level of the PVN unless there is a widespread diffusion of hormone away from the pellet, which we have not observed and would likely result in very low levels of hormone in distant brain sites.

Our data indicate that ERa activation in the peri-PVN induces an increase in neuronal activation within the PVN following stress with DEX pretreatment as measured by the induction of *c-fos* mRNA expression. This argues for a site of action contained to the immediate surround of the PVN. Unfortunately, we currently do not know if there are changes in GAD mRNA and protein levels in ERa-expressing cells within the peri-PVN region following ER agonist treatment in the presence or absence of DEX and/or a stressor. Nor do we know if there are direct projections of these neuronal populations to the PVN. Unfortunately, such studies are technically difficult given the close association of peri-PVN neurons to the PVN proper. It should be mentioned that studies have indicated positive and negative effects of systemically administered estradiol on restraint-induced *c-fos* expression in the PVN (Lund et al., 2004; Figueiredo et al., 2007). This may be due to extrinsic actions

of estradiol on the HPA axis through either ERα or ERβ. However, similar to the results described here, local administration of estradiol to the PVN has been shown to augment *c-fos* expression (Lund et al., 2006).

While it appears from these studies that the inhibitory effects of estradiol on glucocorticoid negative feedback are mediated through ERα, we did observe that the activation of ERβ in and around the PVN results in a slight but significant suppression in HPA responsivity. DEX suppression was not impaired by ERβ activation. Therefore, it can be proposed that ERβ acts in an opposing fashion to increase glucocorticoid-mediated negative feedback. Further experiments would need to be done to establish a dose-response curve for DEX in the presence or absence of ER agonists. ERβ is expressed in a small number of CRH and AVP containing parvocellular PVN neurons, suggesting a potential direct effect on CRH and AVP transcription (Laflamme et al., 1998; Hrabovszky et al., 2004; Suzuki and Handa, 2005). Furthermore, ERβ has been shown to drive CRH and AVP promoter activity *in vitro* (Shapiro et al., 2000; Miller et al., 2004; Pak et al., 2007). Alternatively, ERβ may act peripherally as it is expressed within corticotrophs of the anterior pituitary and within the adrenal cortex (Mitchner et al., 1998; de Cremoux et al., 2008). However, our studies report decreased stress responsivity with local peri-PVN administration of an ERβ agonist, which argues against this possibility.

Impairment of glucocorticoid negative feedback is a key characteristic of major depressive disorder in humans (for reviews see; (Swaab et al., 2005; Bao et al., 2008)). Depressed patients have a marked reduction in ability to respond and adapt to a stressor, and exhibit increased neuropeptide levels in the PVN. Furthermore, in these individuals, DEX is unable to suppress the stimulatory actions of CRH (DEX/CRH test), in much the same fashion as those animals treated with estradiol and PPT and subjected to a restraint stress. Interestingly, normal healthy women have higher cortisol responses to a DEX/CRH test than normal healthy men, regardless of age (Heuser et al., 1994). These data may provide a link between the higher incidence of depression and stress sensitivity observed in women.

CONCLUSION

In summary, estradiol directly impairs glucocorticoid-mediated negative feedback through a peri-PVN ERα dependent mechanism. These data suggest that the increased stress sensitivity seen in females might be due to decreased sensitivity to glucocorticoid-mediated HPA axis negative feedback.

Acknowledgments

Abbreviations

ACTH   adrenocorticotropic hormone

AVP   arginine vasopressin

BnST   bed nucleus of the stria terminalis

CBG	corticosterone binding globulin
CORT	corticosterone
CRH	corticotropin releasing hormone
CSF	cerebrospinal fluid
DAB	3,3′-diaminobenzidine
DEX	dexamethasone
DPN	diarylpropionitrile
DST	dexamethasone suppression test
EB	estradiol benzoate
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
GAD	glutamic acid decarboxylase
GR	glucocorticoid receptor
HPA	hypothalamic-pituitary-adrenal
IR	immunoreactivity
MPOA	medial preoptic area
MR	mineralocorticoid receptor
OVX	ovariectomized
PPT	propylpyrazoletriol
PVN	paraventricular nucleus
RBA	relative binding affinity
RIA	radioimmunoassay
SD	Sprague–Dawley

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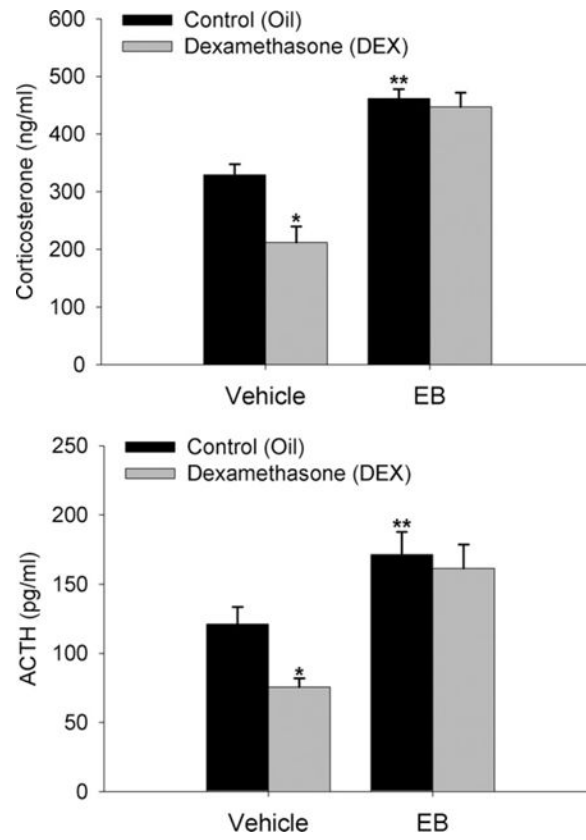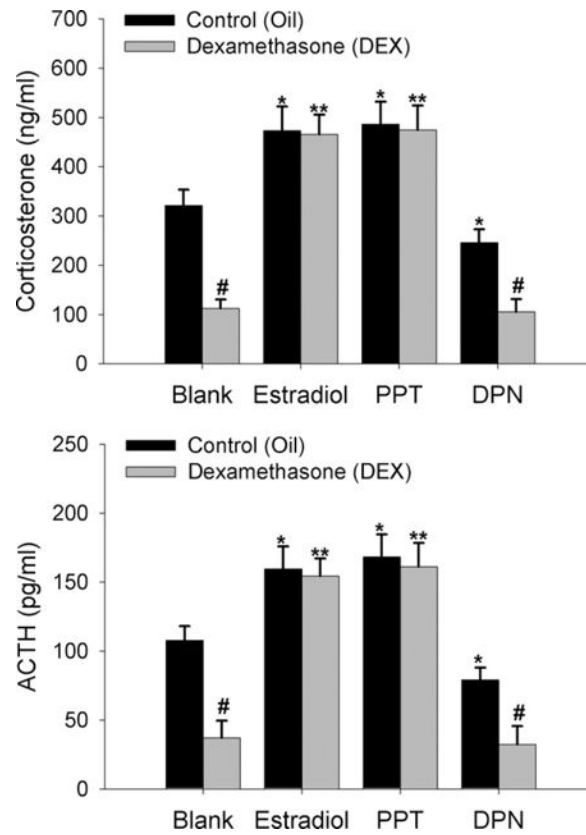
Fig. 1.
Estradiol impairs DEX suppression of diurnal peak plasma CORT and ACTH. Data are expressed as mean±SEM of seven animals per group. *, ** Indicate significant differences from control animals (*P*<0.01).
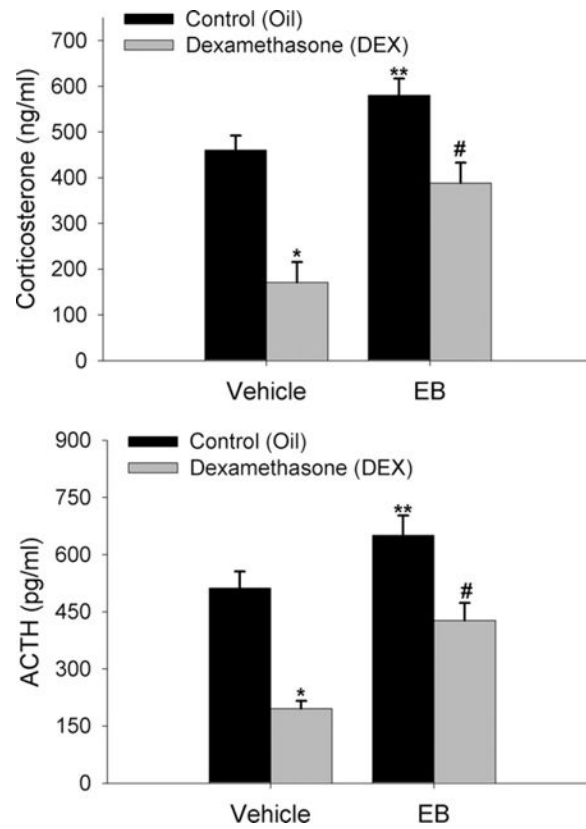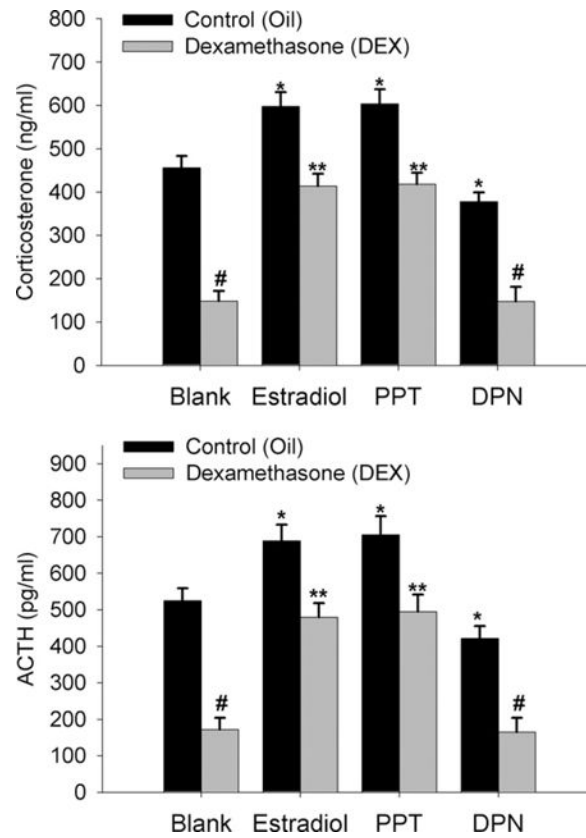
Fig. 2.
Estradiol impairs DEX suppression of plasma CORT and ACTH following a 20 min restraint stress. Data are expressed as mean±SEM of seven animals per group. *, ** Indicate significant differences from control animals ($P<0.01$). # indicates significant difference from DEX treated control animals ($P<0.01$).
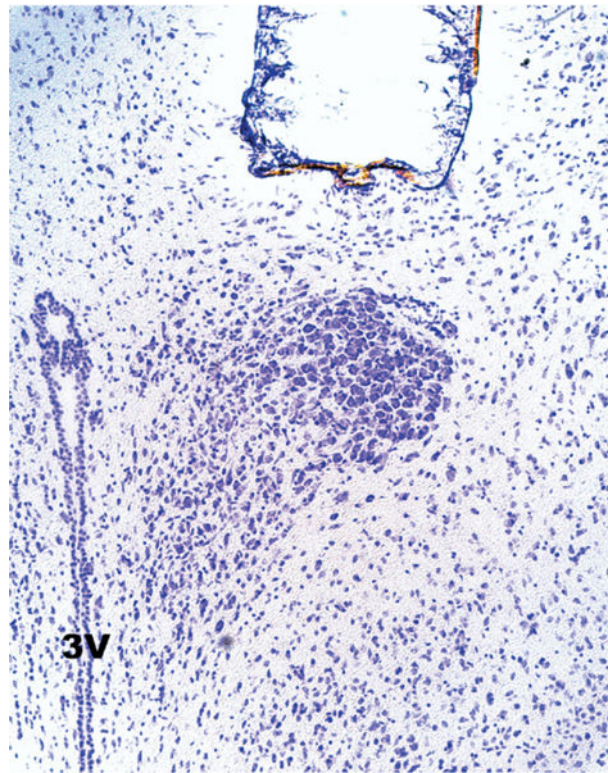
Fig. 3.
Representative photomicrograph of Cresyl Violet–stained tissue indicating location of stereotaxically implanted wax pellet.

Fig. 4.

Estradiol impairs DEX suppression of peak diurnal CORT and ACTH through activation of ERα near the PVN of the hypothalamus. Wax pellets containing estradiol, the ERα agonist PPT, the ERβ agonist DPN, or no hormone (control, blank) were implanted to a region just above the PVN. Data are expressed as mean±SEM of 10 animals per group. *, # Indicate significant differences from oil-treated blank control animals ($P<0.01$). ** Indicates significant difference from DEX-treated control animals ($P<0.01$).
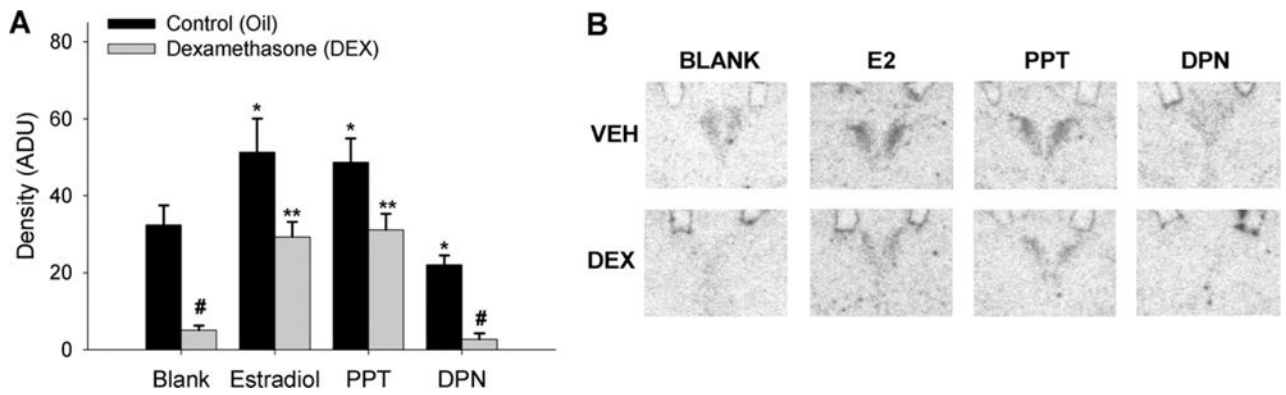
Fig. 5.
Estradiol impairs DEX suppression of restraint-induced CORT and ACTH through activation of ERα near the PVN of the hypothalamus. Wax pellets containing estradiol, the ERα agonist PPT, the ERβ agonist DPN, or no hormone (control, blank) were implanted to a region just above the PVN. Data are expressed as mean±SEM of six to seven animals per group. *, # Indicate significant differences from oil-treated blank control animals ($P<0.01$). ** Indicates significant difference from DEX-treated control animals ($P<0.01$).

Fig. 6.

Estradiol impairs DEX suppression of restraint-induced *c-fos* mRNA expression in the PVN of the hypothalamus through activation of ERα near the PVN. Wax pellets containing estradiol, the ERα agonist PPT, the ERβ agonist DPN, or no hormone (control, blank) were implanted to a region just above the PVN. (A) Relative levels of *c-fos* mRNA expression. Data are represented as mean arbitrary density units ±SEM. *N*=6–7 animals per treatment group. *, # Indicate significant differences from oil-treated blank control animals (*P*<0.05). ** Indicates significant difference from DEX-treated control animals (*P*<0.01). (B) Representative film autoradiograms.
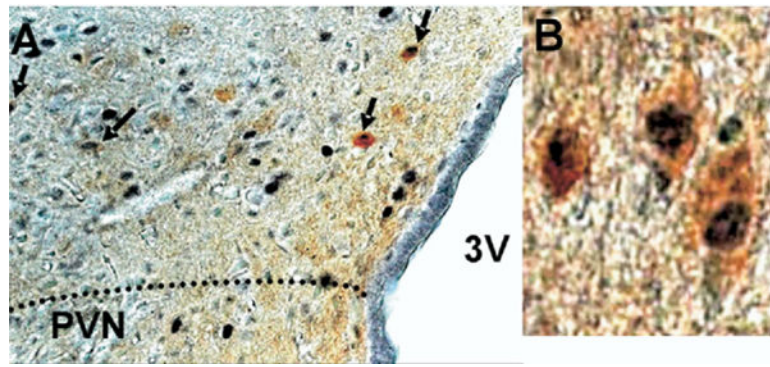
Fig. 7.
GAD67 is colocalized with ERα in the dorsal peri-PVN region (A); 67.4±6.1% of ERα containing neurons also expressed GAD67 in this region. Cells expressing ERα (dark nuclear staining) and GAD67 (brown cytoplasmic staining) (B).