Stress and the female brain. The effects of estradiol on the neurobiological reactions to chronic stress.
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Cyclic estradiol replacement attenuates stress-induced c-Fos expression in the PVN of ovariectomized rats

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Chapter 3

Abstract

Estradiol modulates stress reactions in female rats. Several studies showed anxiolytic effects of estradiol in behavioral tests, but the underlying mechanisms are still unclear. The aim of the current study was to explore how estradiol-treated rats respond to acute and chronic stress compared to ovariectomized rats. Ovariectomized rats received vehicle or 17β-estradiol injections (10 µg/250 g) once every 4 days, which induced alternating high and low plasma 17β-estradiol levels. Stress was presented by daily exposure to an adverse environment in which the animals received 5 footshocks for either 3 or 22 days. Under control conditions no differences were observed, but as soon as stress was applied, reactions of ovariectomized and estradiol-treated rats diverged. Both acute and chronic stress increased the c-Fos protein expression in the paraventricular nucleus of the hypothalamus (PVN). Cyclic estradiol treatment reduced this stress-induced activation of the PVN, an effect that seems to be dependent on the plasma estradiol levels. No differences in stress-induced corticosterone responses were revealed between the treatment groups. An increase in the number of ERβ-expressing cells in the PVN of ovariectomized and estradiol-treated rats during chronic stress implied increased ERβ-mediated mechanisms during these conditions. The dampening effect of estradiol on the excessive stress-induced activity in the PVN may be beneficial for the animal in its response to chronic recurrent stress by reducing the output of the PVN.
Introduction

Severe instabilities in plasma estrogen levels in women, occurring postpartum and during the beginning of the perimenopausal period, are typically associated with an increased risk of the emergence of depressive symptoms and anxiety (Arpels, 1996; Halbreich et al., 2001). Several studies, although not all, have reported a reduction of the depressive symptoms in peri- and postmenopausal women during treatment with estrogen, estrogen-like compounds or estrogen combined with progesterone (Khoo et al., 1998; Carranza-Lira et al., 1999; Strickler et al., 2000; Soares et al., 2001; Friebely et al., 2001; Paoletti et al., 2001; Davis, 2002). In addition, estrogen replacement effectively augmented the effects of selective serotonin re-uptake inhibitors in women suffering from therapy-resistant major depression (Rasgon et al., 2002). Ovariectomized female rats treated with estrogen display a decrease in anxiety-related behavior in repeated open field exposure and reduced immobility in the forced swim test (Rachman et al., 1998; Bowman et al., 2002). Additionally, female rats in proestrus, when the plasma estrogen levels are high, spent more time in the open arms of a plus maze than rats in diestrus (Marcondes et al., 2001). The anxiolytic effects of estradiol are specifically attributed to estrogen receptor β (ERβ) (Walf et al., 2004; Lund et al., 2005). Together, these findings show that estrogen is involved in the modulation of mood and emotion.

Estrogen actions are mediated by interactions with nuclear estrogen receptors (ER), these complexes can bind to estrogen response elements (ERE) that are present in the promotor region of target genes which subsequently modulate gene transcription (McEwen, 2001; Gruber et al., 2002). ERs, appearing as the nuclear ERα and ERβ subtype, are widely expressed in brain areas involved in processing emotional stimuli, for example in the amygdala, the hippocampus, the hypothalamus, the frontal cortex and the raphe nuclei of both human and rats (Laflamme et al., 1998; Osterlund et al., 2001; Shughrue et al., 2001; Gundlah et al., 2001; McEwen, 2001).

Epidemiological studies show that stressful life experiences are associated with the onset of affective disorders, like major depression and anxiety disorders (Post, 1992; Kendler et al., 1995). It has been reported by our group and by others that brain activity in the limbic system and hippocampal areas of rats is affected by chronic stress exposure (Post, 1992; Kaufman et al., 2000; McEwen, 2000; Trentani et al., 2002; Kuipers et al., 2003). Accordingly, chronic stress exposure is used as an animal model for studying the development and treatment of affective disorders.

Estrogen modulates the reactivity of the hypothalamic-pituitary-adrenal (HPA)-axis, the primary neuroendocrine stress response (Viau et al., 1991; Burgess et al., 1992; Carey et al., 1995; Paulmyer-Lacroix et al., 1996; Isgor et al., 2003a). The paraventricular nucleus of the hypothalamus (PVN) is an important relay nucleus in the HPA-axis and, in rodents, mainly expresses ERβ (Shughrue et al., 1997; Laflamme et al., 1998; Shughrue et al., 2001). The occurrence of ERβ in the PVN might serve as an important link in the interaction between estrogen and stress reactivity. Likewise, critical influences of ERβ on the HPA-axis response to stress have been reported (Isgor et al., 2003a).
In the current study, we investigated how several components of the HPA system of ovariectomized female rats that were treated with 17β-estradiol (the most common naturally occurring estrogen) respond to stress compared to ovariectomized rats treated with vehicle. We studied the effects of acute and chronic stress and estradiol treatment on the plasma corticosterone response, ERβ expression and PVN activation. We hypothesized that estradiol-treated rats exhibit more controlled responses to stress than the ovariectomized vehicle-treated rats.

Most studies investigating the effects of estrogen treatment in the central nervous system employed estradiol-releasing pellets or daily injection schemes resulting in invariable, often supraphysiological, plasma estradiol levels. Such invariable levels are likely to induce ER downregulation limiting the possibility for estradiol to interact with its receptor and consequently reducing the binding of ER-estradiol complexes to EREs, which can lead to reduced effects of estradiol in the brain. In our view, cyclic availability of estradiol is highly relevant for a system that is designed to react to alternating high and low levels of estradiol. Natural changes in the plasma estradiol levels may be important for the supportive effects in the regulation of the emotional system. Throughout the estrous cycle, the ratio in plasma gonadal hormones changes daily. We have never observed synchronization of the estrus cycle in previous experiments with cyclic female rats, and moreover some studies claim an effect of stress on the plasma estradiol concentration throughout the cycle (Galea et al., 1997; Shors et al., 1999). To ensure identical estradiol levels in each estradiol-treated rat in the current study we introduced an artificial estradiol cycle. We used a cyclic injection scheme of estradiol administration (every fourth day, comparable to the rat estrus cycle) that produces plasma levels that are within the physiological range, to explore the effects of stress exposure in rats with alternating high and low plasma estradiol levels compared to ovariectomized rats.

**Material and methods**

**Experimental paradigm**

Eighty-seven female Wistar rats (Harlan, Horst, The Netherlands) were individually housed and kept on a reversed 12 hour light-dark cycle with lights on at 7:00 PM. Food and water was available *ad libitum*. The rats were handled and weighed daily. All experimental procedures were performed in the dark phase of the cycle. The experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the protocols were approved by the Animal Experimental Committee of the University of Groningen, The Netherlands (FDC 2698).

All rats were bilaterally ovariectomized (OVX) under isoflurane anesthesia and received once every 4 days at 10:00 AM an injection of either vehicle (OVX; 0.1 ml peanut oil, s.c.) or 17β-estradiol benzoate (E2; 10 µg/250 g in 0.1 ml peanut oil, s.c.) starting at day 3 post-OVX. Body weights were recorded daily between 8:00 and 10:00 AM before the stress session of that day.
The animals were divided into stress and control groups. Chronic stress was presented by a footshock paradigm for 22 days as described earlier (Gerrits et al., 2003) starting on day 10 post-OVX. When the stress was presented acutely, the stress-paradigm was performed in the same manner but lasted only for 3 days. Briefly, the rats were placed in an adverse environment presented by a box with a metal grid floor in which they received 5 footshocks (0.8 mA, 8 sec) per day. Each footshock was preceded by a 5 sec during light stimulus. By presenting only the light stimulus in the adverse environment on the last day, which is strongly associated with stress, only stress-induced responses in the central nervous system become activated whereas pain-induced responses are avoided. The stressor was presented in a randomized manner; the interval between the shocks (3-30 min), the start (8:30 AM-5:00 PM) and duration of each session (15-180 min) and the order of the animals was varied every day. The randomization of the shocks and the adverse environment, triggered by the odor and ultrasonic vocalization of the subsequently stressed rats, add a psychological component to the physical footshock. Control rats were handled daily but were not exposed to the adverse environment.

**Experiment 1: Plasma E2 levels**

To verify alternating high and low plasma levels in the “once in 4 days” injection scheme, the course of the E2 plasma levels was measured following a single injection at day 3 post-OVX in 4 ovariectomized rats. Blood samples were taken 6, 24, 48, 72 and 144 hours after an injection of 10 μg/250 g 17β-estradiol benzoate. Under isoflurane anesthesia the tail was warmed between two surgical heating pads for 1 minute before a tiny tip of the tail was cut off (Fluttert et al., 2000). 300 μl blood was collected in heparinized vials at 4 °C. Subsequently the tail wound was cauterized. The blood samples were separated by centrifugation (3000 rpm for 10 min at 4 °C). A commercially available radioimmunoassay was performed according to the manufacture’s instructions to detect the plasma hormone concentration (ultra sensitive estradiol RIA, DSL-4800; Diagnostic Systems Laboratories Inc., Webster, TX, USA), with a minimum detection limit of 2.2 pg/ml.

**Experiment 2: Plasma CORT response**

The second experiment included 22 rats: 12 vehicle-treated rats (6 OVX-control, 6 OVX-stress) and 10 E2-treated rats sacrificed 24 hours after the last E2 injection (5 E2(24)-control and 5 E2(24)-stress) used to determine the corticosterone (CORT) response at several days throughout a 22 day stress period. Blood samples for CORT determination were taken on the same days the animals received either a vehicle or an E2 injection starting at day 3 post-OVX (day -6, -2, 2, 6, 10, 18 and 22) in the late dark phase between 3:00 and 4:00 PM using the tail bleeding method as described above. The time in the shock box and the interval between the shocks was variable.
on blood sample days, however the last shock of that session was always 15 minutes prior to blood sampling. Immediately after removing the rat from the shock box CORT levels were determined. Control animals were kept in their home cages until the blood sample was taken. Total CORT was extracted from 100 μl plasma and determined by high performance liquid chromatography with UV detection at 254 nm (Dawson, Jr. et al., 1984). Briefly, plasma samples were deproteinized with methanol and centrifuged. The supernatant was further cleaned by extraction on a C8 Solid Phase Extraction Column (Baker, Deventer, The Netherlands). CORT was eluted with acetone and this extract was aspirated and redissolved in 25 % acetonitrile for subsequent injection onto the column (Nucleosil 100-5 C18 l=10 cm, I.D. 3 mm, particle size 5 μm; Chrompack, Middelburg, The Netherlands). Dexamethasone was used as internal standard. The minimum detection limit was 1 mg/dl.

**Experiment 3: PVN activation after acute an chronic stress**

In the third experiment we examined the PVN activation by use of c-Fos expression after acute and chronic stress. The acute stress experiment consisted of 23 rats: 11 vehicle-treated rats (5 OVX-control and 6 OVX-stress) and 12 E2 treated rats sacrificed 24 hours after the last E2 injection (6 E2(24)-control) and 6 E2(24)-stress). The stress was applied for 3 consecutive days. Three days was the minimal time for acute stress in this model because we wanted to avoid electric shocks prior to the perfusion on the third day; the rats should associate the shock box with very unpleasant sensations. The chronic stress experiment consisted of 48 rats: 24 vehicle-treated rats (12 OVX-control and 12 OVX-stress), 12 E2 treated rats sacrificed 24 hours after the last E2 injection (6 E2(24)-control) and 6 E2(24)-stress) and 12 E2 treated rats sacrificed 48 hours after the last E2 injection (6 E2(48)-control and 6 E2(48)-stress). The stress was applied for 22 consecutive days.

**Immunohistochemistry**

On day 3 or on day 22 (depending on the used stress protocol) the rats were put in the stress box for 30 minutes with only the light signal. Two hours later the rats were anesthetized with isoflurane and were transcardially perfused with 0.9 % saline, followed by 300 ml 4 % paraformaldehyde (in 0.1 M phosphate buffer, pH 7.4). We chose a time interval of 2 hours between the perfusion and the last stressor because the expression of c-Fos protein is maximal at this time point (Kovacs, 1998). The brains were removed and postfixed overnight in 4 % phosphate-buffered paraformaldehyde. After cryoprotection in 30 % sucrose 40 μm coronal sections were cut on a cryostat microtome. Immunohistochemical procedures were performed on free-floating sections. c-Fos immunohistochemistry was performed as described earlier (Gerrits et al., 2003). A
rabbit polyclonal antibody raised against c-Fos (Ab-5; Oncogene Research Products, Calbiochem-Novabiochem Int., San Diego, CA, USA; 1:10,000 in 0.01 M phosphate buffered saline (PBS) with 0.25 % Triton and 3 % normal goat serum) was used as the primary antibody while a biotinylated goat anti rabbit IgG (Vector Laboratories, Inc., Burlingame, CA, USA; 1:1000 in 0.01 M PBS) served as the secondary antibody.

ERβ immunohistochemical staining was performed based on the protocol described by Shughrue and Merchenthaler (Shughrue et al., 2001). The sections were treated with 0.2 % Triton X-100 (3 x 10 minutes). After the Triton treatment the sections were incubated in 0.1 M glycine for 30 minutes (in 0.01 M PBS, pH 7.4). Between each treatment the sections were washed in several changes of 0.01 M PBS (pH 7.4). Subsequently the sections were 30 minutes blocked in 1 % bovine serum albumin (BSA) and 1 % H2O2 in 0.01 M PBS and incubated for 72 hours at room temperature with an affinity purified rabbit polyclonal antisem raised against amino acid 468-485 of ERβ (Z8P; Zymed Laboratories, San Francisco, CA, USA, 1:300 in 0.01 M PBS containing 1 % BSA). Cross reactivity of this antiserum for ERβ was excluded by the supplier specifications. This antiserum recognizes both occupied and unoccupied forms of the receptor (Suzuki et al., 2004). A biotinylated goat anti-rabbit IgG served as secondary antibody (Vector, 1:500, in 0.01 M PBS containing 1 % BSA).

The immunoreactivity was revealed with a standard ABC method (Vectastain ABCkit, Vector) followed by a 10 minute DAB-Ni reaction producing black deposits (Gerrits et al., 2003). Thereafter the slices were mounted on gelatin-coated slides, airdried, dehydrated and coverslipped with DEPEX.

To demonstrate colocalization of ERβ and oxytocin (OT), sections were stained for ERβ as described above followed by an incubation of 36 hours at room temperature with rabbit polyclonal antibody raised against OT (Diasorin Inc., Stillwater, MN, USA, 1:5000 in 0.01 M PBS containing 0.3 % triton and 3 % BSA). Cross reactivity of this antibody for vasopressin was excluded by the supplier specifications. Biotinylated goat anti-rabbit IgG served as secondary antibody (Vector, 1:500, in 0.01 M PBS containing 1 % BSA). The immunoreactivity of this reaction was also established with a standard ABC method followed by a 10 minute DAB reaction providing brown cytoplasm deposits.

The c-Fos and ERβ positive cells in the PVN (Bregma –1.08 to –2.00) were quantified using a computerized image analysis system (Leica Qwin version 2.3, Leica Microsystems Imaging Solutions, Cambridge, UK). In order to make objective comparisons, counting was done in a single focus plane using the same magnification and identical gray scale settings as a correction for background staining. The number c-Fos and ERβ positive cells were expressed as number of positive cells/mm2. The c-Fos data were reported as the stress-induced activation compared to control rats. No left-right asymmetry of immunoreactivity was found and therefore the mean ± SEM for both sides was calculated.
Statistical analysis

Results are expressed as the mean ± SEM. Statistical analyses were done with SPSS (version 10.0); p<0.05 was considered significant. Adrenal and uterus weights, the total weight gain during the stress period and the number of c-Fos or ERβ positive cells were analyzed with a two-way ANOVA with treatment (OVX or E2) and protocol (control or stress) as between subject variables followed by an LSD pairwise comparison when applicable. The course of the plasma E2 concentration and CORT response data were analyzed using a repeated measures ANOVA with time as within subject variable and, in case of the CORT data, treatment (OVX or E2) and protocol (control or stress) as between subject variables followed by a LSD pairwise comparison. Sphericity assumed modeling, with Greenhouse-Geisser and Huynh-Feldt adjustments, was applied (Quintana et al., 1994).

Results

A single injection with 10 μg/250 g BW of 17β-estradiol benzoate induced plasma levels of 31.1±4.16 pg/ml measured 6 hours after the injection. This level declined significantly to 19.9±1.73 pg/ml after 24 hours (p=0.042) and to 10.8±0.58 pg/ml after 48 hours (p=0.026). 72 hours after the injection the plasma reached a concentration of 8.7±1.43 pg/ml (p=0.018) and remains constant at this level (Figure 1). The plasma 17β-estradiol concentration of OVX rats 5 weeks after the surgery was 10.5±0.58 pg/ml. The lower E2 concentrations accounted for significantly reduced uterus weights in OVX rats (0.08±0.005 g) compared to E2-treated rats (0.57±0.021 g; F_{1,9}=433.81, p<0.001).

Cyclic E2 treatment had a significant main effect on weight gain (F_{1,18}=18.43, p<0.001; Figure 2A). During control conditions E2-treated rats gained less weight in a period of 3 weeks than OVX animals (F_{1,18}=16.51, p=0.001). Stress reduced weight gain (F_{1,18}=18.53, p<0.001). This reached significance in the OVX rats (F_{1,18}=18.10, p<0.001), but not in the E2-treated rats (F_{1,18}=3.67, p=0.071).
E2-treated rats did not display different basal plasma CORT concentrations compared to OVX rats. Stress had a significant effect on the CORT response of OVX and E2-treated rats ($F_{1,17}=8.918$, $p=0.008$; Figure 2B). Stress significantly increased the plasma CORT levels on day 2 in the OVX (up to 49 μg/100ml; $p=0.012$) and the E2-treated rats (up to 58 μg/100ml; $p=0.001$). The CORT response in E2-treated rats was similar to OVX rats. Moreover, in both experimental groups the plasma CORT concentration returned to baseline levels within the first week of the stress protocol. In contrast, there was a significant effect of chronic stress on the adrenal glands of these rats after 22 days ($F_{1,18}=9.58$, $p=0.006$; Figure 2C). OVX rats displayed adrenal hypertrophy after 3 weeks of stress ($F_{1,18}=8.39$, $p=0.010$), while chronic stress exposure had no significant effect on the adrenal weight of E2-treated rats ($F_{1,18}=2.39$, $p=0.139$).

Acute stress induced a dramatic increase in c-Fos expression in the PVN of OVX rats ($F_{1,17}=164.05$, $p<0.001$). After 3 weeks of stress there is still a significant increased expression of c-Fos compared to control rats ($F_{1,19}=29.51$, $p<0.001$), however, the intensity is much lower compared to the response to acute stress ($F_{2,28}=36.14$, $p<0.001$; Figure 3). The stress-induced c-Fos activation after both acute and chronic stress was significantly affected by E2 treatment ($F_{1,28}=21.09$, $p<0.001$).
E2-treated animals showed a significant reduction of the c-Fos-activation compared to OVX rats when sacrificed 24 hours after the last E2 injection ($F_{1,28}=16.92$, $p<0.001$ and $F_{1,28}=7.05$, $p=0.013$ respectively). This effect was dependent on the E2 plasma level on the day of sacrifice, since animals sacrificed 48 hours after the last injection (Figure 1), when E2 plasma concentrations were markedly reduced, demonstrated a non-significant reduction of the chronic stress-induced c-Fos expression ($F_{1,28}=1.42$, $p=0.24$). During control conditions there were no differences found in c-Fos expression in the PVN between OVX and E2-treated rats.

**Figure 3**: A) Stress-induced activation of the PVN measured by Fos expression after acute and chronic stress in OVX and E2 treated rats. The animals were sacrificed 24 hours (24; high plasma E2 concentration) or 48 hours (48; low plasma E2 concentration) after the last E2 injection. Microphotographs of the PVN in B) OVX-control, C) E2(24)-control, D) OVX-acute stress and E) E2(24)-acute stress rats. * $p<0.05$ compared to OVX, # $p<0.05$ compared to the accompanying acute stress group; 3V=third ventricle.

**Figure 4**: A) Microphotograph of ERβ immunoreactive nuclei in the dorsal and ventral subdivision of the medial parvocellular area and the lateral subdivision of the magnocellular area of the caudal PVN; 3V=third ventricle. B) Oxytocin and ERβ double immunoreactive cells in the PVN; oxytocin=grey cytoplasm, ERβ=black nuclei. C) Magnification of the square in panel B; black arrows=double labeled cell, arrowhead=oxytocin immunoreactive cell.
ERβ labeling in the PVN was found mostly in the dorsal and ventral subdivision of the medial parvocellular area and the lateral subdivision of the magnocellular area of the caudal part (Bregma –1.78 to –2.00; Figure 4A). The expression of ERβ in OT-ergic neurons is demonstrated in Figure 4B with a magnification in Figure 4C. Acute stress did not alter the number of ERβ expressing cells ($F_{1,19}=1.14$, $p=0.30$). Chronic stress, however, slightly increased the number of cells in the PVN expressing ERβ protein ($F_{1,19}=4.30$, $p=0.052$; Figure 5). Nevertheless, post-hoc evaluation revealed no statistical differences within the OVX and E2-treated groups. Cyclic E2 treatment did not affect the basal level of number of cells expressing ERβ in the PVN.

**Discussion**

In this study we described a different approach to study the effects of estrogen on the brain. Most studies investigating the effects of E2 administration on the brain often use E2 releasing pellets or daily injections. Pellets are commercially available and easy to use but have the major disadvantage that the plasma E2 levels remain relatively constant and at a high concentration. Such experimental designs will give information about E2 effects in short during studies, but will not accurately display E2 effects at physiological conditions in long during studies. The major disadvantage of these setups is that ER expression may be changed due to the constantly high E2 levels, which consequently might influence the effects of E2 on the nervous system.

In the current study administration of 10 µg/250 g bodyweight 17β-estradiol benzoate once every 4 days resulted in physiological plasma estradiol levels (below 50 pg/ml) with alternating high and low levels (Figure 1). After 96 hours, when the next injection will occur, the plasma E2 levels were maximally declined. The OVX rats displayed levels of plasma E2 comparable to the low levels achieved in the E2-treated rats and also showed extremely decreased uterus size, which ensured successful surgeries.

The significant reduction in growth rate and the adrenal hypertrophy in chronically stressed OVX rats (Figure 2) indicate that these rats have suffered from the stressor during the whole period of 3 weeks. Stress-induced adrenal hypertrophy is a reversible process; we
observed normalized adrenal weights after 3 weeks of recovery of the stress (unpublished observation). E2 itself has an inhibitory effect on weight gain as shown by a strong increase in bodyweight after OVX, which has also been described before (Geary et al., 1999). E2-treated rats did not show stress-induced changes in growth rate and adrenal size. OVX rats display a strong increase in the CORT response during the first days of the stress paradigm, an effect corresponding to a dramatically increased c-Fos expression in the PVN.

However, if the stressor is persistent, though presented in a randomized paradigm, an adaptation is shown in the CORT response, an effect also shown in the PVN by a lowered but still significant c-Fos expression. Although the CORT response is faded, adrenal hypertrophy is still observed after 22 days. As shown before in our lab, stress effects on adrenal weight were not attributable to changes in medulla volume but to an increased volume of the adrenal cortex, the tissue responsible for CORT synthesis (Kuipers, 2004). Three weeks of stress with a highly psychological component did induce a c-Fos response in the PVN of OVX rats, although lower than found in acutely stressed rats, while rats subjected to chronic restraint stress show total habituation of the c-Fos response in the PVN (Stamp et al., 1999). Therefore, there seems only to be a partial habituation to the currently used stress paradigm, although we were not able to detect increased CORT levels after 3 weeks of stress, the PVN still showed activation to some level and the adrenals were still enlarged. The missing correlation between CORT levels and PVN activation might indicate dissociation between the PVN and the HPA-axis during recurrent stress; however, based on the adrenal hypertrophy this is unlikely.

CORT levels increase according to the intensity of the stimulus; however, this rise is also dependent on the phase of the circadian cycle of the animal (Retana-Marquez et al., 2003). Nocturnal animals like the rat have the highest plasma CORT levels at the early dark phase of the cycle, which are declining during the night to rather low plasma CORT levels during the day (Allen-Rowlands et al., 1980). It has been reported that stress has maximal impact on CORT levels when applied in the early light phase of the cycle when baseline CORT concentrations are lowest (Retana-Marquez et al., 2003). The current study was performed under a reversed light-dark regimen, which might explain the controversial results between plasma CORT levels and adrenal weights. Other studies of our group presented stress during the light phase and did reveal increased CORT levels in female rats after 22 days (Kuipers, 2004).

E2-treated rats displayed the same CORT response to acute and chronic stress as OVX rats. However, in contrast to our results, most studies reported higher CORT responses to stress in E2-treated rats compared to OVX (Burgess et al., 1992; Lunga et al., 2004; Lund et al., 2005). Besides showing higher responses to stress, also a longer release of CORT has been reported, a characteristic we might have missed by only taking blood samples immediately after the stressor. Most of these studies were performed in rats replaced with invariable high plasma levels of E2, which is markedly different to our variable plasma levels. Likewise, Carey et al. (Carey et al., 1995) reported no different CORT response to a novel cage after a single injection of 10 µg E2 compared to OVX rats.
Cyclic 17β-estradiol administration within the physiological range reduced the impact of stress exposure on PVN activation. Moreover, if the exposure to the adverse environment is within 24 hours after the last E2 injection (when the E2 level is still high) the reduction of c-Fos expression in the PVN is more pronounced. This result implies that the intensity of the stress-induced PVN activation depends on the plasma E2 concentration and runs parallel to the obliged E2 cycle.

E2 mediates its actions, among other pathways, through the nuclear receptors ERα and ERβ. The PVN in the rat expresses high levels of ERβ in contrast to almost no expression of ERα (Shughrue et al., 1997; Simonian et al., 1997; Laflamme et al., 1998; Shughrue et al., 2001). The distribution pattern of ERs in this brain area however, shows large species differences, the PVN in humans expresses more ERα than ERβ (Osterlund et al., 2000). The expression pattern of ERβ protein within the PVN as revealed in the current study corresponds to the described localization in the literature. The most intense nuclear labeling of ERβ positive cells in the PVN is observed in the dorsal and ventral subdivision of the medial parvocellular area and the lateral subdivision of the magnocellular area of the caudal PVN (Simonian et al., 1997; Hrabovszky et al., 2004).

Acute stress did not change the number of cells expressing ERβ in the PVN. On the other hand, chronic stress slightly increased the number of ERβ positive cells in the PVN in both treatment groups, which suggest that ERβ-mediated effects increase in the PVN after long term stress exposure. Remarkably the increased receptor expression is observed in both the OVX and E2-treated rats, implying that the availability of the ligand is not a critical factor in this process. Recently it has been reported that glucocorticoids induce ERβ expression (Isgor et al., 2003a; Suzuki et al., 2004). Therefore, the increased expression of ERβ in our study might be a consequence of elevated CORT levels induced by chronic stress. Cyclic E2 administration did not induce a change in the number of ERβ-expressing cells in the PVN compared to OVX rats, a result that is supported by a study using a single dose of E2 (Greco et al., 2001). However, other studies have reported decreased ERβ expression in the PVN of E2-treated rats with invariable E2 levels (Patisaul et al., 1999; Suzuki et al., 2004). These findings indicate that the E2 administration paradigm is crucial in the regulatory properties on the receptor level.

One possible mechanism of E2 in decreasing the PVN activation induced by stress might involve an interaction with oxytocin (OT). ERβ is among other neuron types expressed in OT-ergic neurons in the ventral subdivision of the medial parvocellular area and the lateral subdivision of the magnocellular area of the caudal PVN (Figure 4) (Simonian et al., 1997; Laflamme et al., 1998; Alves et al., 1998; Hrabovszky et al., 1998; Isgor et al., 2003b; Hrabovszky et al., 2004). The OT gene contains a functional ERE (Richard et al., 1990; Mohr et al., 1991; Adan et al., 1993; Loven et al., 2001). The colocalization of ERβ and OT implies a significant role for ERβ in the E2 dependent regulation of OT release by the PVN. Indeed, treatment of wildtype or ERβ knockout mice with E2, induced increased OT mRNA expression in the PVN only in the wildtype mice (Nomura et al., 2002; Patisaul et al., 2003).
It has been reported that OT infused locally into the PVN repressed the c-Fos response to a stressor which might explain our reduced c-Fos expression in stress-exposed E2 treated rats (Windle et al., 2004). Hypothetically, activation of ER\(\beta\) by E2 will increase the OT gene transcription. Consequently dendritic release of OT protein in the PVN will decrease the stress-induced c-Fos expression. Also the E2 plasma level dependent stress-induced c-Fos response can be explained through this mechanism, showing high repression of the c-Fos activation with high plasma E2 levels and low repression with low plasma E2 levels.

In conclusion, as shown in the current study, estradiol depletion as in the OVX rats is accompanied by decreased uterus size and increased weight gain. The other studied parameters like adrenal weight, CORT levels, c-Fos and ER\(\beta\) expression in the PVN did not show differences during control conditions. However, as soon as stress is applied to these rats there is a divergence in the reactions. Cyclic 17\(\beta\)-estradiol benzoate administration reduced the activation of the PVN induced by stress dependent on the plasma concentration. Moreover, rats treated with E2 did not show stress-induced growth reduction and adrenal hypertrophy. In this study no differences in stress-induced CORT responses were revealed between OVX and E2-treated rats. The dampening of the excessive stress-induced activity in the PVN due to estradiol may be beneficial for the animal in its response to chronic repetitive stress by reducing the output of the PVN. In general, if 17\(\beta\)-estradiol is able to reduce the unwanted effects of stress in more areas of the central nervous system besides the PVN, than 17\(\beta\)-estradiol (or a specific ER\(\beta\) agonist with less peripheral side-effects) may prevent the deleterious consequences of chronic stress on total brain function.

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