Available online at www.sciencedirect.com
SCIENCE DIRECT
Hormones and Behavior 49 (2006) 197 – 205
Hormones and Behavior
www.elsevier.com/locate/yhbeh

Estrogen facilitates fear conditioning and increases corticotropin-releasing hormone mRNA expression in the central amygdala in female mice

Aaron M. Jasnow [a,*], Jay Schulkin [b,c], Donald W. Pfaff [a]

[a] Laboratory of Neurobiology and Behavior, Box 275, The Rockefeller University, 1230 York Avenue, New York, NY 10021, USA
[b] Department of Physiology and Biophysics, Georgetown University School of Medicine, Washington, DC 20007, USA
[c] Clinical Neuroendocrinology Branch, NIMH, DHHS, Bethesda, MD 20892, USA

Received 21 February 2005; revised 21 June 2005; accepted 28 June 2005
Available online 3 August 2005

Abstract

Estrogens exert important actions on fear and anxiety-like behavior both in humans and non-human animals. Currently, the mechanisms underlying estrogenic modulation of fear are not known. However, evidence suggests that estrogens may exert their influence on fear within the amygdala. The purpose of the present study was to examine effects of estrogen on fear conditioning. Specifically, the present study examined whether long-term estrogen treatment in ovariectomized female mice via Silastic capsule implantation would facilitate both contextual and cued fear conditioning. In a separate set of experiments, we then examined whether estrogen treatment in ovariectomized female mice would modulate corticotropin-releasing hormone (CRH) gene expression within the amygdala. Long-term estrogen treatment facilitated both contextual and cued fear. Ovariectomized mice treated with estrogen froze significantly more to a context as well as to a discrete auditory cue. In addition, estrogen treatment significantly increased CRH mRNA expression within the central nucleus of the amygdala as measured by in situ hybridization and quantitative PCR. These data raise the possibility that estrogens could influence fear responses in females through their actions in the amygdala.
© 2005 Elsevier Inc. All rights reserved.

Keywords: Hormones; Anxiety; Stress; Emotion

The amygdala and its afferent and efferent projections are important for the acquisition and expression of conditioned fear (LeDoux, 2000; Davis, 2000). Whereas the neural circuitry underlying fear conditioning in mammals has been well characterized, relatively little is known about the actions of estrogens within the amygdala and how they may modulate fear. Evidence suggests that changes in circulating levels of estrogens are linked to alterations in emotion as well as to the development of mental illness in females (Campbell and Whitehead, 1977; Sichel et al., 1995; Arpels, 1996; Sherwin, 1998). Both α-estrogen receptor (ERα) and β-estrogen receptor (ERβ) genes are widely distributed throughout the amygdala in both rodents and humans (Osterlund et al., 1998, 2000; Shughrue et al., 1998; Laflamme et al., 1998; Merchenthaler et al., 2004), suggesting that this region is a likely site at which estrogens exert their actions on fear.

Importantly, it is not known how activation of estrogen receptors within the amygdala becomes translated into changes in fear. One possibility is that the neuropeptide corticotropin-releasing hormone (CRH) serves as an intermediary between estrogens and alterations in fear. In addition to its actions on the HPA axis (Koob, 1992; Valentino and Van Bockstaele, 2002), CRH occupies extra-hypothalamic nuclei throughout the central nervous system and mediates behavioral, autonomic and immune responses to stressors independent of the HPA axis (Brown and Fisher, 1983; Koob et al., 1984; Eaves et al., 1985; Britton et al., 1986; Dunn and Berridge, 1990; Pavcovich and Valentino, 1997; Moreau et al., 1997; Jones et al., 1998).

* Corresponding author. Fax: +1 212 327 8664.
E-mail address: jasnowa@rockefeller.edu (A.M. Jasnow).

0018-506X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.yhbeh.2005.06.005

The central amygdala is thought to coordinate several of the actions of CRH on physiological and behavioral responses to aversive stimuli. CRH mRNA and protein levels are increased in the central amygdala following an aversive training stimulus of a brief footshock (Makino et al., 1999; Roozendaal et al., 2002). In addition, administration of CRH receptor antagonists, either intracerebroventricularly or within discrete brain regions, blocks the behavioral effects of exposure to a stressor and to exogenously administered CRH (Menzaghi et al., 1994; Fendt et al., 1997; Mansbach et al., 1997; Lee and Davis, 1997; Jasnow et al., 1999, 2004).

Interestingly, the human CRH gene contains half-palindromic estrogen-responsive elements (ERE), through which direct estrogenic transcriptional regulation has been demonstrated, in vitro (Vamvakopoulos and Chrousos, 1993), and an indirect pathway has also been suggested (Miller et al., 2004). CRH mRNA is also increased in the ventral paraventricular nucleus (PVN) of female rats on the day of proestrus when estrogen is at its peak (Bohler et al., 1990). Taken together, these data suggest that CRH regulates, in part, physiological and behavioral responses to aversive stimuli, and that the central amygdala coordinates many of these responses. Moreover, these data suggest that the CRH gene is under direct transcriptional regulation by estrogens, thereby providing a potential mechanism within the brain through which estrogens modulate emotional learning. If true, then exogenous estrogen should facilitate fear learning as well as increase CRH gene expression within the central nucleus of the amygdala. The present study examined whether estrogen treatment would facilitate fear learning in female mice as well as modulate CRH gene transcription within the central nucleus of the amygdala.

Materials and methods

Animals and housing conditions

In the fear conditioning experiments, adult female Swiss Webster mice (*Mus musculus*) were obtained from Taconic Farms. Animals were housed individually for 10 days prior to behavioral testing in a temperature-controlled (20 ± 2°C) colony room on a 12:12-h light–dark cycle with lights off at 11:00 h. All animals were housed in Plexiglas cages (26.5 cm × 16 cm × 20 cm) with corn cob (Bed-O-Cob) bedding, with wire mesh tops, and food and water were available ad libitum. In the in situ hybridization and quantitative PCR experiments, animals were group housed (5 animals/cage) in the same colony room, and food and water were available ad libitum. All procedures and protocols were approved by The Rockefeller University Institutional Animal Care and Use Committee (IACUC), and The Public Health Services Policy on Humane Care and Use of Laboratory Animals was strictly followed.

Behavioral apparatus

All training and testing occurred in a mouse test cage (Coulbourn Instrument, Allentown, PA) located in a sound-attenuating cabinet in an isolated testing room. The test cage was constructed of modular aluminum panels (two sidewalls) and Plexiglas (rear wall and front wall) with a test cage shock floor for delivering the footshock unconditioned stimuli (US). Two speakers were mounted on one of the modular side panels, one for delivering the tone conditioned stimulus (CS) and one for delivering white noise. An infrared activity monitor was mounted 7 in. above the shock floor in order to monitor freezing behavior. Freezing was only scored if the mouse was immobile for at least 1 s. All behavioral training and testing occurred in the dark period of the daily L–D cycle. During all training sessions, a small light producing ~32 lx illuminated the test cage and white noise (65 dB) was presented throughout the procedure. A ventilation fan provided air circulation and additional background noise. The test cage was cleaned between each animal with 100% ethanol during the training and contextual testing session and cleaned with 1% acetic acid during the cued fear testing session.

Effects of estrogens on contextual fear

Twenty animals were used in this experiment. Animals were received from Taconic Farms ovariectomized and were housed in a colony room for 1 week prior to experimental manipulation. Animals were matched by weight and assigned to 1 of 2 experimental conditions. Animals in the first group were surgically implanted with a Silastic capsule containing estradiol benzoate (EB) (Sigma), whereas the second group received a Silastic capsule containing vehicle control (sesame oil). Capsules consisted of 2 mm long Silastic tubing (0.078 in ID × 0.125 in OD) closed at both ends with medical grade silicone adhesive (Dow Corning). EB was suspended in sesame oil at a concentration of 1 μg/μl and injected into the Silastic capsule. This dose has previously been used by our lab to investigate non-reproductive behaviors in mice and produces a proestrus level of circulating estrogen (Morgan and Pfaff, 2001, 2002). Animals were housed individually for 10 days prior to behavioral testing. On the first day, mice were introduced to the conditioning chamber and allowed to explore for 5 min, then placed back their home cage and immediately returned to the colony room. Twenty-four hours later mice were placed in the same conditioning chamber for 3 min and then received a single 1-s, 0.5-mA shock. Sixty seconds later, animals were removed from the conditioning chamber and placed back in their home cages and immediately returned to the colony room. Twenty-four hours after the conditioning procedure, animals were tested for contextual fear in the same conditioning chamber under the same conditions for 8 min. Freezing behavior was

measured in 1-min epochs throughout the 8-min testing session.

Effects of estrogens on cued fear

Forty animals were used for the cued fear experiment. On the first day mice were introduced to the conditioning chamber and allowed to explore for 5 min. Twenty-four hours later animals were placed in the conditioning chamber for 3 min and were then presented with three 30-s tones (75–80 dB) each coterminating with a 1-s, 0.5-mA footshock with an inter-tone interval of 60 s. Twenty-four hours later, fear to the tone conditioned stimulus was measured by placing the mice in a novel environment that was different than the training cage and presenting three 30-s tones (75–80 dB), 2 min and 30 s after being placed in the test cage with a 100-s inter-tone interval. During the cued fear test, the overhead light, as well as the white noise, was turned off. Freezing was scored during the tone presentation and during the baseline period before presentation of the first tone as described above.

Effects of estrogens on CRH gene expression in the CeA: in situ hybridization

Animals (n = 16) were received from Taconic Farms ovariectomized and housed in a colony room for 1 week prior to experimental manipulation. The mice were matched by weight and assigned to 1 of 2 experimental conditions. The first group received a subcutaneous (s.c.) injection of EB (5 μg dissolved in 0.1 ml sesame oil), whereas the second group received vehicle control (sesame oil). Twenty-four hours after the first injection, the first group received an s.c. injection of EB (10 μg), whereas group 2 again received vehicle control. Two hours after this final injection, animals were killed; their brains were removed, rapidly frozen on dry ice and stored at −80°C until sectioning. The brains were cut into 12-μm sections with a cryostat at −20°C and directly mounted onto electrostatically charged glass microscope slides. Sections were processed and hybridized with an ^{35}S-labeled riboprobe to CRH mRNA as described below.

To construct the CRH probe, total RNA was isolated from an adult Swiss Webster mouse brain with Trizol reagent (Invitrogen, San Diego, CA) according to the manufacturer's instructions. The RNA was then reverse transcribed into single-stranded cDNA using SuperScript Choice (Invitrogen, San Diego, CA). CRH-specific primer pairs were designed based on the rat cDNA sequence from GenBank (Accession no.NM_031019) with the aid of DS Gene (Accelrys, San Diego). The primer sequences were as follows: forward 5′-CAAGCTCAC-AGCAACAGGAAACTG-3′, reverse 5′-CAACTGGG-TGACTTCCATCTGCTT-3′. PCR amplification of the cDNA yielded a single band of 373 bp corresponding to nucleotides 695–1068 of rat CRH. The fragment was purified and subsequently used for the generation of a riboprobe to CRH mRNA. The template for the CRH probe was constructed by ligating a T7 promoter site directly to the PCR fragment with the aid of the Lig'nScribe Kit (Ambion, Austin, TX). This procedure was used for both the sense and antisense templates. The T7 antisense and sense templates were used to prepare cRNA probes labeled with ^{35}S-UTP using the Maxiscript (Ambion, Austin, TX) T7 in vitro transcription reagents. The sections were processed according to the protocol outlined in Simeone (1998). Briefly, slide mounted sections were thawed and fixed in 4% paraformaldehyde for 15 min. Sections were then processed with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min and washed 3 times in 2× SSC for 3 min and then 50% formamide/2× SSC for 5 min. Prior to hybridization, the sections were dehydrated in a graded series of ethanol solutions (50%, 70%, 95%, 100%) and processed with chloroform for 5 min. Sections were incubated in hybridization buffer (50% deionized formamide, 0.3 M NaCl, 20 mM Tris–HCl, pH 7.5, 5 mM EDTA, pH 8.0, 10% dextran sulfate, 1× Denhardt's solution and 1 mg/ml tRNA) with ^{35}S-labeled CRH antisense or sense cRNA probes for 16 h at 55°C. After hybridization, the slides were exposed to stringent washes, treated with RNase A, dehydrated and subjected to autoradiography for 13 days.

Effects of estrogen on CRH expression in the amygdala: quantitative real-time PCR

In order to more accurately determine estrogen's effect of CRH gene expression, another group of animals (n = 60) was used for Experiment 3. Animals were matched by weight and assigned to 1 of 3 groups. The first group was administered a single s.c. injection of EB (10 μg) or vehicle control (sesame oil) and killed 2 h later. The second group also received a single s.c. injection of EB (10 μg) or vehicle control but were killed 24 h later. The final group of animals was surgically implanted with a Silastic capsule containing either EB or vehicle control, in the same manner as in Experiment 1, and were killed 10 days later. In all groups, brains were rapidly removed, frozen on dry ice and stored at −80°C until sample collection. Brains were mounted in a cryostat and sliced to the area of interest. A 1-mm diameter area encompassing the central, as well as part of the lateral and basolateral amygdala, was dissected out bilaterally using a stainless steel punch tool, deposited into separate 1.5 ml microcentrifuge tubes at −20°C and then stored at −80°C until RNA isolation. RNA was isolated using Trizol reagent according to the manufacturer's instructions (Invitrogen, San Diego, CA) and purified using the Qiagen RNeasy mini kit (Qiagen, Valencia, CA).

The total RNA isolated from mouse amygdala was precisely assayed using the Agilent Bioanalyzer 2100

(Agilent Technologies, Palo Alto, CA) and with a UV spectrophotometer. Then, 400 ng of total RNA from each sample was reverse transcribed using ABI Taqman reverse transcription reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Briefly, each reaction contained 400 ng total RNA, 1× reverse transcription buffer, 5.5 mM $MgCl_2$, 500 μM of each dNTP, 2.5 μM oligo $d(T)_{16}$, 0.4 U/μl RNase inhibitor and 1.25 U/μl Multiscribe Reverse Transcriptase. The cycling conditions for the reverse transcription reaction were as follows: 25°C for 10 min, 48°C for 30 min and 95°C for 5 min. Following thermal cycling all samples were diluted with nuclease-free H_2O, 1:15 cDNA: H_2O and stored at −20°C until assayed. The primers for quantitative PCR were designed using PRIMER EXPRESS® software (Applied Biosystems). The forward and reverse primers were as follows: forward: 5′-GTTGAATTTCTTGCAA-CCGGAG-3′; reverse: 5′-GACTTCTGTTGAGGTTCC-CCAG-3′, yielding an amplicon of 101 bp. A standard curve method was used to quantify cDNA levels during quantitative PCR according to Bustin (2000, 2002). In order to construct standard curves, mouse cDNA was PCR amplified using the above primers which yielded a single fragment of 101 bp corresponding to nucleotides 295–395 of rat CRH. The fragment was purified and subsequently rapidly TA ligated into the PCR®2.1-TOPO plasmid according to the manufacturer's instructions (Invitrogen, San Diego, CA). The resulting clone was re-analyzed for size and presence of unique restrictions sites, and then linearized. A standard calibration curve was then prepared in which the cloned CRH sequence was present at 3.0×10^5–3.0×10^0 copies and then stored at −80°C until assayed. Quantitative PCR was conducted on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using SYBR green dye on the experimental samples and the standard calibration curve, in triplicate on a single 96-well plate for each group.

Statistical analyses

In the fear conditioning experiments, freezing observations were transformed into a percentage of total time and analyzed using a Mann–Whitney *U* test in the case of the contextual fear and with a repeated measures analysis of variance (ANOVA) with treatment as the between-subjects factor and the response to the tone as the within-subjects factor for the cued fear experiment. Main effects were further analyzed with a Tukey–Kramer post hoc test. Baseline freezing in the auditory fear experiment was analyzed with a Student's *t* test. Pre- and post-shock freezing was also analyzed with a Student's *t* test. For the in situ hybridization experiment, optical density was analyzed with the aid of MCID image analysis system (Imaging Research, St. Catherine's ON, Canada). For each animal, the optical density obtained represented the average of measurements taken from 2 to 3 sections from both the left and right amygdala and analyzed using a Student's *t* test. For the qPCR experiments, an average was taken from the triplicate reactions for each sample and analyzed using a Student's *t* test. For all statistical analyses, significance was ascribed at $P < 0.05$.

Results

Estrogen treatment facilitates contextual fear

Figs. 1a and b show that there was a significant effect of hormone treatment on freezing to the context ($P < 0.05$). Specifically, EB-treated mice froze significantly more during the 8-min testing session compared with vehicle-treated mice. In addition, there were no differences in pre-shock ($t_{18} = 1.779$, $P > 0.05$; Fig. 2a) and post-shock freezing between EB-treated and vehicle-treated animals ($t_{18} = 0.530$, $P > 0.05$; Fig. 2b).

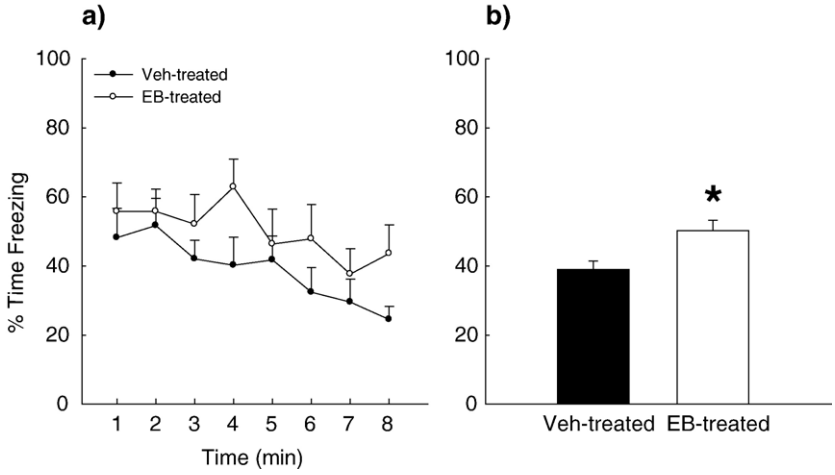

Fig. 1. Mean (±SEM) percent time freezing to a context during each minute of an 8-min test (a) and over the entire 8-min test (b) of female mice implanted with Silastic capsules containing either estradiol benzoate (EB) or vehicle. Mice were trained with a single 0.5-mA shock and tested for freezing to the context 24 h later. Significant differences between groups are denoted by an asterisk (*) ($P < 0.05$).

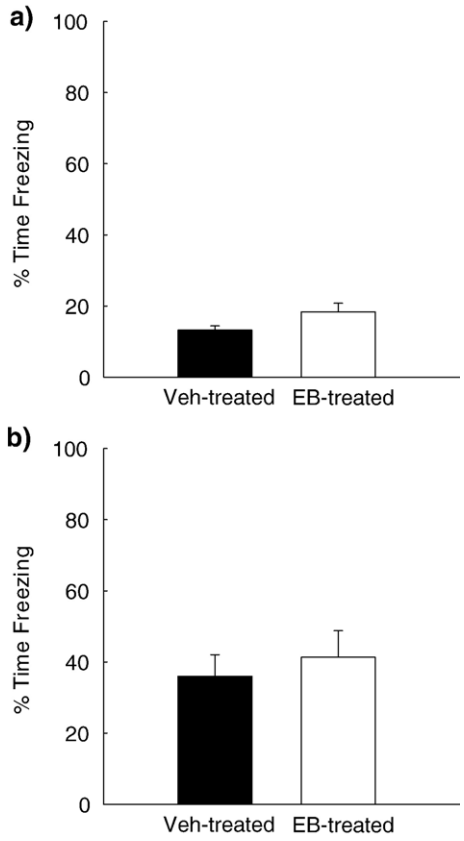

Fig. 2. (a) Mean (±SEM) percent time freezing during the time period preceding the footshock administration. There were no differences in pre-shock freezing between EB-treated and vehicle-treated animals ($P > 0.05$). (b) Mean (±SEM) percent time freezing during the time period immediately following footshock administration. There were no differences in post-shock freezing between EB-treated and vehicle-treated animals ($P > 0.05$).

Estrogen treatment facilitates cued fear

There was no difference in baseline freezing between EB-treated and vehicle-treated animals ($t_{39} = 1.690$, $P > 0.05$). There was a main effect of hormone treatment ($F_{(1,117)} = 5.15$, $P < 0.05$) and a main effect of tone presentation ($F_{(1,117)} = 4.53$, $P < 0.05$) on freezing, but no significant interaction effect ($F_{(1,117)} = 0.11$, $P > 0.05$). Specifically, mice that were treated with EB froze significantly more during the tone presentation than mice treated with vehicle (Fig. 3).

Estrogen treatment increases CRH mRNA expression in the central amygdala: in situ hybridization

Fig. 4a shows coronal sections through the amygdala exposed to autoradiographic film. Sections treated with sense probes did not have significant signal. There was a significant effect of treatment on CRH mRNA expression in the central nucleus of the amygdala ($t_{14} = 4.67$, $P < 0.05$). Specifically, mice that were administered EB had significantly greater CRH mRNA expression within the central amygdala compared with vehicle-treated mice (Figs. 4a and b).

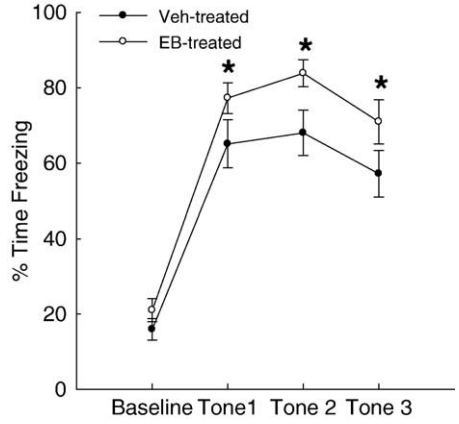

Fig. 3. Mean (±SEM) percent time freezing during the baseline period and during cue presentation. Mice froze significantly more during the presentation of the tone conditioned stimulus (cue) compared with during the baseline freezing period. EB treatment significantly increased freezing to a tone conditioned stimulus compared with animals treated with vehicle control. Significant differences are denoted by an asterisk (*).

Although, it has been reported in previous studies, we did not observe a significant increase in CRH gene expression in the PVN of EB-treated animals compared with vehicle-treated animals in the present study (data not shown).

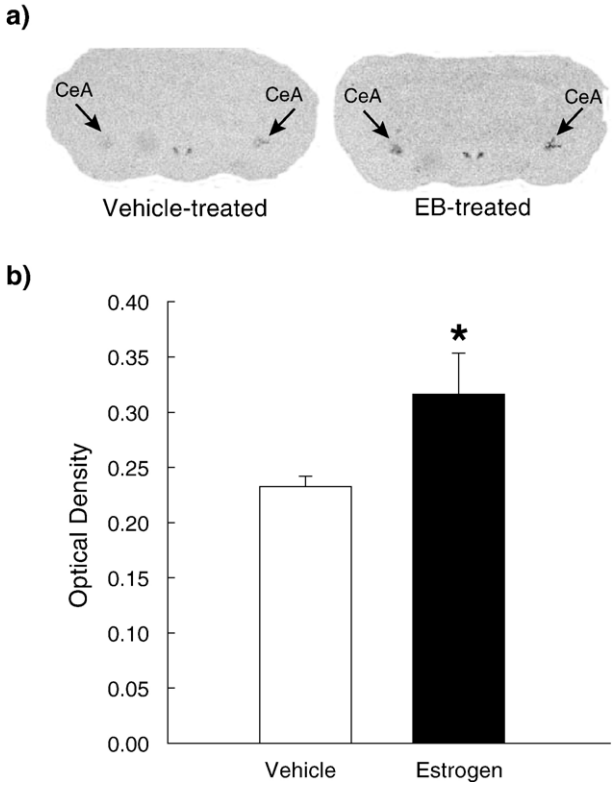

Fig. 4. Film autoradiographs from an ^{35}S-labeled probe revealed expression of CRH mRNA in the central amygdala (CeA) and paraventricular nucleus (PVN) (a). Ovariectomized (OVX) EB-treated mice displayed significantly more CRH mRNA expression within the CeA compared with OVX vehicle-treated mice (a, b). Animals were administered a subcutaneous injection of 5 μg of EB and then 24 h later administered 10 μg and killed 2 h after this final injection. Significant differences are denoted by an asterisk (*).

Estrogen treatment increases CRH mRNA expression in the central amygdala: quantitative real-time PCR

The standard curve for the 2-h animals was $r^2 = 0.999$; for the 24-h animals was $r^2 = 0.981$; and for the 10-day animals was $r^2 = 0.998$. The qPCR runs yielded a single product as analyzed by a melting curve and by gel electrophoresis (Fig. 5). In addition, qPCR on the no-RT control samples did not amplify any product demonstrating the absence of genomic DNA contamination. There was no significant effect on CRH gene expression either 2 h after EB treatment or after 10 days of capsule EB treatment ($t_{16} = 0.079$, $P > 0.05$, Fig. 6a; $t_{17} = 1.67$, $P > 0.05$, Fig. 6c, respectively). In contrast, there was a significant effect on CRH gene expression 24 h after EB treatment ($t_{15} = 5.65$, $P < 0.05$, Fig. 6b). Specifically, mice that were treated with EB displayed greater levels of CRH mRNA compared with vehicle-treated mice. Therefore, we have characterized the CRH response to estrogen treatment within the amygdala by a second approach as well, demonstrating that peak transcript levels appear 24 h after EB treatment.

Discussion

The present data demonstrate that estrogen treatment facilitates fear conditioning to both contextual and discrete cues in female mice. Animals that received Silastic capsules containing EB displayed significantly elevated freezing during testing in response to a contextual as well as to a tone cue compared with vehicle-treated animals. In addition, the present data indicate that estrogen treatment modulates CRH gene expression within the central nucleus of the amygdala. Mice that received a subcutaneous injection of EB displayed increased CRH mRNA expression in the

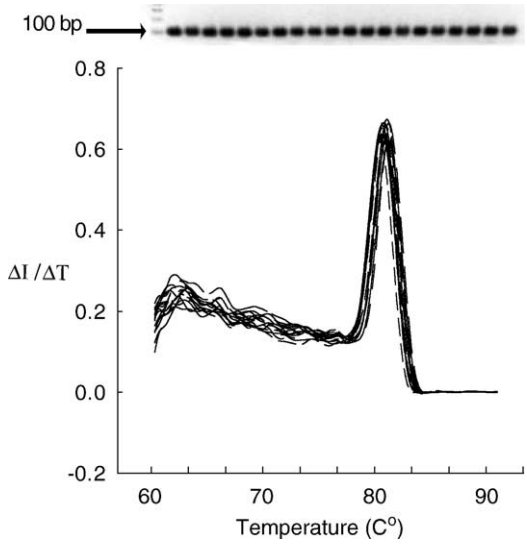

Fig. 5. CRH gene expression was determined by quantitative PCR. The resulting reaction was run on a 2% agarose gel to determine the size a specificity of the amplicon. Representative electrophoretic lanes of the PCR reaction produced a single amplicon that was approximately 100 bp, as predicted. In addition, a dissociation analysis of the CRH PCR reaction demonstrated that there was a single amplicon with a melting temperature equivalent to that predicted by the PRIMER EXPRESS software. This indicates that the fluorescent signal was used to quantify CRH amplification and was due to a single, specific product. (ΔI/ΔT = change in fluorescence intensity over the change in time).

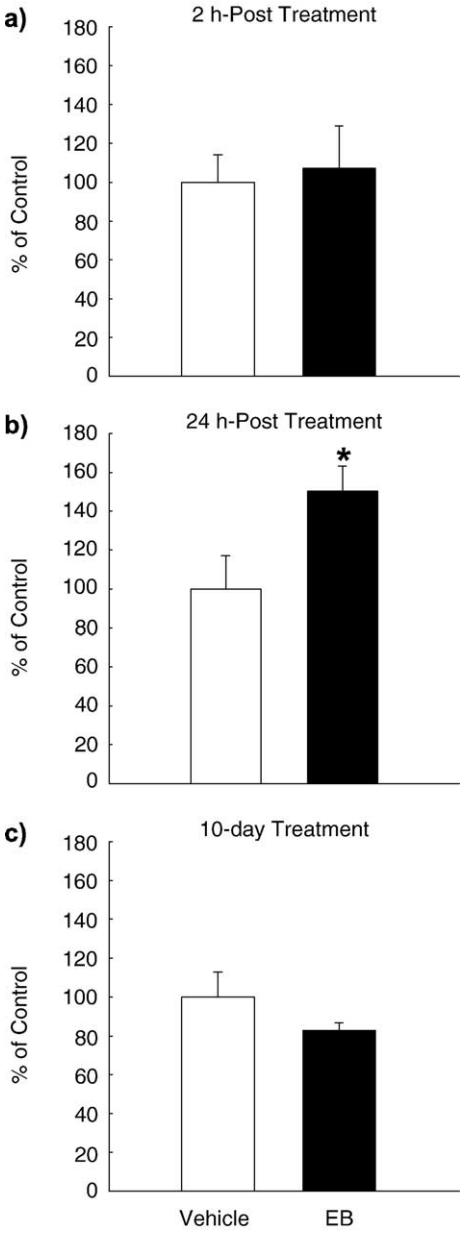

Fig. 6. Mean (±SEM) of CRH mRNA levels in the amygdala from EB-treated and vehicle-treated mice killed 2 h (a), 24 h (b) after treatment or after 10 days of treatment with a Silastic capsule (c). 400 ng of total RNA from amygdala was analyzed by quantitative real-time PCR using the primers and reaction conditions as described above. mRNA copy numbers were calculated from the CRH standard curve generated simultaneously. CRH gene expression was then transformed into percent of control (vehicle-treated mice) per 400 ng of total RNA. EB treatment significantly increased CRH gene expression in the amygdala 24 h after treatment, but not 2 h after treatment and not after 10 days of treatment compared with vehicle-treated animals. Significant differences are denoted by an asterisk (*).

central amygdala as measured by in situ hybridization and qPCR. These data suggest that estrogens can modulate fear conditioning in female mice as well as CRH gene expression within the central amygdala, a region that is important for fear conditioning.

The findings of the present study are consistent with a number of previous reports that demonstrate the ability of estrogens to modulate aversive learning. For instance, elevated estrogen levels during proestrus are associated with a facilitation of conditioned avoidance responses in a two-way active avoidance task in rats (Sfikakis et al., 1978). A recent report demonstrated that women at high estrogen stages during the menstrual cycle display increased fear recognition compared with women at low estrogen stages (Pearson and Lewis, 2005). Likewise, estrogen treatment facilitates trace eyeblink conditioning (Leuner et al., 2004), retention of a passive avoidance task (Morgan and Pfaff, 2002) and freezing to a conditioned stimulus (Morgan and Pfaff, 2001) in rats and mice. Thus, evidence suggests that estrogens have a facilitative effect on various forms of aversive learning. There are also reports, however, that suggest estrogens have an inhibitory effect on aversive learning. For instance, one report found estrogen-treated female rats to have lower retention scores compared with non-treated rats on a passive avoidance task (Gibbs et al., 1998). Other studies have found high estrogen levels or estrogen treatment to be associated with decreased contextual conditioning (Markus and Zecevic, 1997b; Gupta et al., 2001), but found no differences in conditioning to a tone (Markus and Zecevic, 1997a). The reasons behind these discrepancies in the literature are not fully apparent.

One possible explanation for the results of the present study is that the increased freezing observed in EB-treated mice could be the result of either a performance deficit in ovariectomized vehicle-treated mice or due to a modulation of footshock sensitivity in EB-treated mice. It is unlikely that the increased freezing observed in EB-treated mice was simply due to a reduction of freezing in vehicle-treated mice as the result of a performance deficit. Vehicle-treated mice displayed normal levels of immediate post-shock freezing on the conditioning day, and also exhibited normal conditional freezing to the context and tone on the test day. In addition, there was no significant difference in freezing prior to administration of the footshock. This is consistent with previous reports (Farr et al., 1995; Gupta et al., 2001; Leuner et al., 2004). It is also unlikely that the increased freezing observed in EB-treated mice in the present study was due to an increase in footshock sensitivity. There was no significant difference in immediate post-shock freezing between EB-treated and vehicle-treated animals in the present study. This is also consistent with several previous reports (Farr et al., 1995; Gupta et al., 2001; Leuner et al., 2004). However, some studies have demonstrated both increased (Drury and Gold, 1978) and decreased (Frye and Walf, 2004) footshock and pain sensitivity following estrogen treatment.

The present study demonstrated that CRH gene expression within the central nucleus of the amygdala is upregulated by EB treatment. Specifically, CRH gene expression was elevated 24 h after EB treatment, but not after 2 h or chronic treatment. During the estrous cycle, females are exposed to increasing levels of circulating estrogens, reaching peak levels during proestrus. Theoretically, the time course of the CRH gene expression response observed in the present study could fit into the context of the natural estrous cycle. With estrogens reaching peak levels on the morning of proestrus, the absence of an early effect might allow the female to engage in courtship behaviors. Following this, the female would become much more conservative and fearful after mating. Currently, it is not clear why CRH gene expression was not increased following 10 days of EB treatment.

It is well known that CRH, in part, regulates physiological, behavioral and autonomic responses to stressors. For example, the physiological and behavioral effects of exogenous administration of CRH or its related peptides are similar to exposure to a natural stressor (Brown et al., 1982; Brown and Fisher, 1983; Koob et al., 1984; Sherman and Kalin, 1987; Dunn and Berridge, 1990; Moreau et al., 1997; Jones et al., 1998). Also, infusions of CRH antagonists block the behavioral effects of exposure to stressors and to exogenously administered CRH (Menzaghi et al., 1994; Korte et al., 1994; Lundkvist et al., 1996; Fendt et al., 1997; Martins et al., 1997; Mansbach et al., 1997). CRH is also involved in learning and memory in both human and non-human animals (Sahgal et al., 1983; Chen et al., 1992; Lee et al., 1992; Behan et al., 1995; Radulovic et al., 1999; Roozendaal et al., 2002). In sheep, estrogen treatment increases CRH mRNA expression within the bed nucleus of the stria terminalis (BNST), but not within the PVN (Broad et al., 1995), similar to the effects demonstrated in the present study.

Although not explicitly tested here, the results of the present study raise the possibility that CRH within the CeA may serve as an intermediary between estrogens and alterations in fear. The design of the experiments, however, does not allow us to conclude that there is a direct mechanistic link between the two systems. Lee and Davis (1997) hypothesized that stress activates CRH-containing neurons within the CeA, which then release CRH within the BNST, an area that contains robust levels of both CRH_1 and CRH_2 receptors (Smagin and Dunn, 2000). In fact, CRH levels have been reported to be increased in the CeA following a footshock training stimulus (Roozendaal et al., 2002), and most studies observe c-*fos* induction in the lateral division of the CeA following exposure to a stressor (Campeau et al., 1997) which is the division of the CeA that sends CRH-containing afferents to the BNST (Sakanaka et al., 1986). CRH-induced modulation of the BNST may then activate projection nuclei, including those in the hypothalamus and brainstem, which might further modulate neuroendocrine and behavioral responses to stressors. The

connection between the CeA and the BNST is just one possible route. The CeA sends projections to a variety of other brain areas involved in regulating changes in the autonomic nervous system and behavior during states of fear. For example, projections from the central nucleus of the amygdala to the lateral hypothalamus influence autonomic and blood pressure changes, whereas projections to the periaqueductal grey influence freezing behavior (LeDoux et al., 1988; Fanselow, 1991). In addition, direct connections with the locus coeruleus regulate increased vigilance and attention during times of stress and may be responsible for release of norepinephrine (Van Bockstaele et al., 1996; Van Bockstaele et al., 1998). CRH effects on learning and memory have also been reported within the basolateral amygdala. For example, local infusion of a CRH antagonist reduces memory consolidation of an inhibitory avoidance task (Roozendaal et al., 2002). It has been suggested that the source of the CRH acting on the basolateral amygdala originates in the CeA (Roozendaal et al., 2002). It is possible that estrogens could modulate the output of the CeA to multiple brain regions involved in learning and memory, but whether this actually occurs is currently unknown.

In summary, the data presented in this report along with previous data provide evidence that estrogens can facilitate fear learning. In addition, the present data demonstrate that estrogens increase CRH mRNA expression within the CeA, a region that is important for regulating fear responses.

Acknowledgments

The authors thank Bob Cuevos and Darrel Merrick for their expert animals care, Par Parekh and Christine Min for their technical assistance and Oliver Fricke for thoughtful discussions during the preparation of the manuscript. This work was supported by MH 070086 to AMJ.

References

Arpels, J.C., 1996. The female brain hypoestrogenic continuum from the premenstrual syndrome to menopause. A hypothesis and review of supporting data. J. Reprod. Med. 41, 633–639.

Behan, D.P., Heinrichs, S.C., Troncoso, J.C., Liu, X.J., Kawas, C.H., Ling, N., De Souza, E.B., 1995. Displacement of corticotropin releasing factor from its binding protein as a possible treatment for Alzheimer's disease. Nature 378, 284–287.

Bohler Jr., H.C., Zoeller, R.T., King, J.C., Rubin, B.S., Weber, R., Merriam, G.R., 1990. Corticotropin releasing hormone mRNA is elevated on the afternoon of proestrus in the parvocellular paraventricular nuclei of the female rat. Brain Res. Mol. Brain Res. 8, 259–262.

Britton, D.R., Varela, M., Garcia, A., Rosenthal, M., 1986. Dexamethasone suppresses pituitary-adrenal but not behavioral effects of centrally administered CRF. Life Sci. 38, 211–216.

Broad, K.D., Keverne, E.B., Kendrick, K.M., 1995. Corticotrophin releasing factor mRNA expression in the sheep brain during pregnancy, parturition and lactation and following exogenous progesterone and oestrogen treatment. Brain Res. Mol. Brain Res. 29, 310–316.

Brown, M.R., Fisher, L.A., 1983. Central nervous system effects of corticotropin releasing factor in the dog. Brain Res. 280, 75–79.

Brown, M.R., Fisher, L.A., Rivier, J., Spiess, J., Rivier, C., Vale, W., 1982. Corticotropin-releasing factor: effects on the sympathetic nervous system and oxygen consumption. Life Sci. 30, 207–210.

Bustin, S.A., 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J. Mol. Endocrinol. 25, 169–193.

Bustin, S.A., 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J. Mol. Endocrinol. 29, 23–39.

Campbell, S., Whitehead, M., 1977. Oestrogen therapy and the menopausal syndrome. Clin. Obstet. Gynaecol. 4, 31–47.

Campeau, S., Falls, W.A., Cullinen, W.E., Helmreich, D.L., Davis, M., Watson, S.J., 1997. Elicitation and reduction of fear: behavioral and neuroendocrine indices and brain induction of the immediate–early gene c-*fos*. Neuroscience 78, 1087–1104.

Chen, M.F., Chiu, T.H., Lee, E.H., 1992. Noradrenergic mediation of the memory-enhancing effect of corticotropin-releasing factor in the locus coeruleus of rats. Psychoneuroendocrinology 17, 113–124.

Davis, M., 2000. The role of the amygdala in conditioned and unconditioned fear and anxiety. In: Aggleton, J.P. (Ed.), The Amygdala. Oxford Univ. Press, Oxford, pp. 213–287.

Drury, R.A., Gold, R.M., 1978. Differential effects of ovarian hormones on reactivity to electric footshock in the rat. Physiol. Behav. 20, 187–191.

Dunn, A.J., Berridge, C.W., 1990. Physiological and behavioral responses to corticotropin-releasing factor administration: is CRF a mediator of anxiety or stress responses? Brain Res. Brain Res. Rev. 15, 71–100.

Eaves, M., Thatcher-Britton, K., Rivier, J., Vale, W., Koob, G.F., 1985. Effects of corticotropin releasing factor on locomotor activity in hypophysectomized rats. Peptides 6, 923–926.

Fanselow, M.S., 1991. The midbrain periaqueductal gray as a coordinator of action in response to fear and anxiety. In: Depaulis, A., Bandler, R. (Eds.), The Midbrain Periaqueductal Gray Matter: Functional, Anatomical and Neurochemical Organization. Plenum Publishing, New York, pp. 151–173.

Farr, S.A., Flood, J.F., Scherrer, J.F., Kaiser, F.E., Taylor, G.T., Morley, J.E., 1995. Effect of ovarian steroids on footshock avoidance learning and retention in female mice. Physiol. Behav. 58, 715–723.

Fendt, M., Koch, M., Schnitzler, H., 1997. Corticotropin-releasing factor in the caudal pontine reticular nucleus mediates the expression of fear-potentiated startle in the rat. Eur. J. Neurosci. 9, 299–305.

Frye, C.A., Walf, A.A., 2004. Estrogen and/or progesterone administered systemically or to the amygdala can have anxiety-, fear-, and pain-reducing effects in ovariectomized rats. Behav. Neurosci. 118, 306–313.

Gibbs, R.B., Burke, A.M., Johnson, D.A., 1998. Estrogen replacement attenuates effects of scopolamine and lorazepam on memory acquisition and retention. Horm. Behav. 34, 112–125.

Gupta, R.R., Sen, S., Diepenhorst, L.L., Rudick, C.N., Maren, S., 2001. Estrogen modulates sexually dimorphic contextual fear conditioning and hippocampal long-term potentiation (LTP) in rats. Brain Res. 888, 356–365.

Jasnow, A.M., Banks, M.C., Owens, E.C., Huhman, K.L., 1999. Differential effects of two corticotropin-releasing factor antagonists on conditioned defeat in male Syrian hamsters (*Mesocricetus auratus*). Brain Res. 846, 122–128.

Jasnow, A.M., Huhman, K.L., Davis, M., 2004. Involvement of central amygdalar and bed nucleus of the stria terminalis corticotropin-releasing factor in behavioral responses to social defeat. Behav. Neurosci. 118, 1052–1061.

Jones, D.N., Kortekaas, R., Slade, P.D., Middlemiss, D.N., Hagan, J.J., 1998. The behavioural effects of corticotropin-releasing factor-related peptides in rats. Psychopharmacology (Berl.) 138, 124–132.

Koob, G.F., 1992. The behavioral neuroendocrinology of corticotropin-releasing factor, growth-hormone releasing factor, somatostatin, and gonadotropin-releasing hormone. In: Nemeroff, C.B. (Ed.), Neuroendocrinology. CRC Press, Ann Arbor, pp. 353–396.

Koob, G.F., Swerdlow, N., Seeligson, M., Eaves, M., Sutton, R., Rivier, J., Vale, W., 1984. Effects of alpha-flupentixol and naloxone on CRF-induced locomotor activation. Neuroendocrinology 39, 459–464.

Korte, S.M., Korte-Bouws, G.A., Bohus, B., Koob, G.F., 1994. Effect of corticotropin-releasing factor antagonist on behavioral and neuroendocrine responses during exposure to defensive burying paradigm in rats. Physiol. Behav. 56, 115–120.

Laflamme, N., Nappi, R.E., Drolet, G., Labrie, C., Rivest, S., 1998. Expression and neuropeptidergic characterization of estrogen receptors (ERalpha and ERbeta) throughout the rat brain: anatomical evidence of distinct roles of each subtype. J. Neurobiol. 36, 357–378.

LeDoux, J.E., 2000. Emotion circuits in the brain. Annu. Rev. Neurosci. 23, 155–184.

LeDoux, J.E., Iwata, J., Cicchetti, P., Reis, D.J., 1988. Different projections of the central amygdaloid nucleus mediate autonomic and behavioral correlates of conditioned fear. J. Neurosci. 8, 2517–2529.

Lee, Y., Davis, M., 1997. Role of the hippocampus, the bed nucleus of the stria terminalis, and the amygdala in the excitatory effect of corticotropin-releasing hormone on the acoustic startle reflex. J. Neurosci. 17, 6434–6446.

Lee, E.H., Hung, H.C., Lu, K.T., Chen, W.H., Chen, H.Y., 1992. Protein synthesis in the hippocampus associated with memory facilitation by corticotropin-releasing factor in rats. Peptides 13, 927–937.

Leuner, B., Mendolia-Loffredo, S., Shors, T.J., 2004. High levels of estrogen enhance associative memory formation in ovariectomized females. Psychoneuroendocrinology 29, 883–890.

Lundkvist, J., Chai, Z., Teheranian, R., Hasanvan, H., Bartfai, T., Jenck, F., Widmer, U., Moreau, J.L., 1996. A non peptidic corticotropin releasing factor receptor antagonist attenuates fever and exhibits anxiolytic-like activity. Eur. J. Pharmacol. 309, 195–200.

Makino, S., Shibasaki, T., Yamauchi, N., Nishioka, T., Mimoto, T., Wakabayashi, I., Gold, P.W., Hashimoto, K., 1999. Psychological stress increased corticotropin-releasing hormone mRNA and content in the central nucleus of the amygdala but not in the hypothalamic paraventricular nucleus in the rat. Brain Res. 850, 136–143.

Mansbach, R.S., Brooks, E.N., Chen, Y.L., 1997. Antidepressant-like effects of CP-154,526, a selective CRF1 receptor antagonist. Eur. J. Pharmacol. 323, 21–26.

Markus, E.J., Zecevic, M., 1997a. Sex differences and estrous cycle changes in hippocampus-dependent fear conditioning. Psychobiology 25, 246–252.

Markus, E.J., Zecevic, M., 1997b. Sex differences and estrous cycle changes in hippocampus-dependent fear conditioning. Psychobiology 25, 246–252.

Martins, A.P., Marras, R.A., Guirnaraes, F.S., 1997. Anxiogenic effect of corticotropin-releasing hormone in the dorsal periaqueductal grey. NeuroReport 8, 3601–3604.

Menzaghi, F., Howard, R.L., Heinrichs, S.C., Vale, W., Rivier, J., Koob, G.F., 1994. Characterization of a novel and potent corticotropin-releasing factor antagonist in rats. J. Pharmacol. Exp. Ther. 269, 564–572.

Merchenthaler, I., Lane, M.V., Numan, S., Dellovade, T.L., 2004. Distribution of estrogen receptor alpha and beta in the mouse central nervous system: in vivo autoradiographic and immunocytochemical analyses. J. Comp. Neurol. 473, 270–291.

Miller, W.J.S., Suzuki, S., Miller, L.K., Handa, R., Uht, R.M., 2004. Estrogen receptor (ER){beta} isoforms rather than ER{alpha} regulate corticotropin-releasing hormone promoter activity through an alternate pathway. J. Neurosci. 24, 10628–10635.

Moreau, J.L., Kilpatrick, G., Jenck, F., 1997. Urocortin, a novel neuropeptide with anxiogenic-like properties. NeuroReport 8, 1697–1701.

Morgan, M.A., Pfaff, D.W., 2001. Effects of estrogen on activity and fear-related behaviors in mice. Horm. Behav. 40, 472–482.

Morgan, M.A., Pfaff, D.W., 2002. Estrogen's effects on activity, anxiety, and fear in two mouse strains. Behav. Brain Res. 132, 85–93.

Osterlund, M., Kuiper, G.G., Gustafsson, J.A., Hurd, Y.L., 1998. Differential distribution and regulation of estrogen receptor-alpha and -beta mRNA within the female rat brain. Brain Res. Mol. Brain Res. 54, 175–180.

Osterlund, M.K., Keller, E., Hurd, Y.L., 2000. The human forebrain has discrete estrogen receptor alpha messenger RNA expression: high levels in the amygdaloid complex. Neuroscience 95, 333–342.

Pavcovich, L.A., Valentino, R.J., 1997. Regulation of a putative neurotransmitter effect of corticotropin-releasing factor: effects of adrenalectomy. J. Neurosci. 17, 401–408.

Pearson, R., Lewis, M.B., 2005. Fear recognition across the menstrual cycle. Horm. Behav. 47, 267–271.

Radulovic, J., Ruhmann, A., Liepold, T., Spiess, J., 1999. Modulation of learning and anxiety by corticotropin-releasing factor (CRF) and stress: differential roles of CRF receptors 1 and 2. J. Neurosci. 19, 5016–5025.

Roozendaal, B., Brunson, K.L., Holloway, B.L., McGaugh, J.L., Baram, T.Z., 2002. Involvement of stress-released corticotropin-releasing hormone in the basolateral amygdala in regulating memory consolidation. Proc. Natl. Acad. Sci. 99, 13908–13913.

Sahgal, A., Wright, C., Edwardson, J.A., Keith, A.B., 1983. Corticotrophin releasing factor is more potent than some corticotrophin-related peptides in affecting passive avoidance behaviour in rats. Neurosci. Lett. 36, 81–86.

Sakanaka, M., Shibasaki, T., Lederis, K., 1986. Distribution and efferent projections of corticotropin-releasing factor-like immunoreactivity in the rat amygdaloid complex. Brain Res. 382, 213–238.

Sfikakis, A., Spyraki, C., Sitaras, N., Varonos, D., 1978. Implication of the estrous cycle on conditioned avoidance behavior in the rat. Physiol. Behav. 21, 441–446.

Sherman, J.E., Kalin, N.H., 1987. The effects of ICV-CRH on novelty-induced behavior. Pharmacol. Biochem. Behav. 26, 699–703.

Sherwin, B.B., 1998. Estrogen and cognitive functioning in women. Proc. Soc. Exp. Biol. Med. 217, 17–22.

Shughrue, P.J., Scrimo, P.J., Merchenthaler, I., 1998. Evidence for the colocalization of estrogen receptor-beta mRNA and estrogen receptor-alpha immunoreactivity in neurons of the rat forebrain. Endocrinology 139, 5267–5270.

Sichel, D.A., Cohen, L.S., Robertson, L.M., Ruttenberg, A., Rosenbaum, J.F., 1995. Prophylactic estrogen in recurrent postpartum affective disorder. Biol. Psychiatry 38, 814–818.

Smagin, G.N., Dunn, A.J., 2000. The role of CRF receptor subtypes in stress-induced behavioural responses. Eur. J. Pharmacol. 405, 199–206.

Valentino, R.J., Van Bockstaele, E.J., 2002. Corticotropin-releasing factor: putative neurotransmitter actions of a neurohormone. In: Pfaff, D.W., Arnold, A.P., Etgen, A.M., Fahrbach, S.E., Rubin, R.T. (Eds.), Hormones, Brain and Behavior. Elsevier, San Diego, pp. 81–102.

Vamvakopoulos, N.C., Chrousos, G.P., 1993. Evidence of direct estrogenic regulation of human corticotropin-releasing hormone gene expression. Potential implications for the sexual dimorphism of the stress response and immune/inflammatory reaction. J. Clin. Invest. 92, 1896–1902.

Van Bockstaele, E.J., Chan, J., Pickel, V.M., 1996. Input from central nucleus of the amygdala efferents to pericoerulear dendrites, some of which contain tyrosine hydroxylase immunoreactivity. J. Neurosci. Res. 45, 289–302.

Van Bockstaele, E.J., Colago, E.E.O., Valentino, R.J., 1998. Amygdaloid corticotropin-releasing factor targets locus coeruleus dendrites: substrate for the co-ordination of emotional and cognitive limbs of the stress response. J. Neuroendocrinol. 10, 743–757.