Effects of Estrogen Treatment on Expression of Brain-Derived Neurotrophic Factor and cAMP Response Element-Binding Protein Expression and Phosphorylation in Rat Amygdaloid and Hippocampal Structures

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Key Words

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Abstract

Clinical studies indicate an effect of estrogen (E₂) on affect and cognition, which may be mediated by the cAMP response element-binding protein (CREB) pathway and CREB-related gene target brain-derived neurotrophic factor (BDNF). We investigated the effect of E₂ on CREB expression and phosphorylation and BDNF expression in the amygdala and hippocampus, areas involved in emotional processing. Ovariectomized rats were given 10 µg 17β-estradiol or vehicle for 14 days and expression of components of the CREB signaling pathway, i.e., CREB, phosphorylated CREB (pCREB), and BDNF in amygdala and hippocampus were investigated using immunogold labeling. Levels of BDNF mRNA were determined by in situ reverse-transcriptase polymerase chain

reaction. We also examined the effect of E₂ on calcium/calmodulin kinase (CaMK IV) immunolabeling in the hippocampus. E₂ increased immunolabeling and mRNA levels of BDNF in the medial and basomedial amygdala and CA1 and CA3 regions of the hippocampus, but not in any other amygdaloid or hippocampal regions examined. E₂ increased immunolabeling of CREB and pCREB in the medial and basomedial, but not central or basolateral amygdala. E₂ also increased CaMK IV and pCREB immunolabeling in the CA1 and CA3 regions, but not CA2 region or dentate gyrus, of the hippocampus. There was no change in immunolabeling of CREB in any hippocampal region. These data identify a signaling pathway through which E₂ increases BDNF expression that may underlie some actions of E₂ on affective behavior and indicate neuroanatomical heterogeneity in the E₂ effect within the amygdala and hippocampus.

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Introduction

Estrogen (E_2) has been shown to have powerful growth effects in neurons [for reviews, see 1–3]. These include effects in areas relevant to reproduction, such as the ventromedial nucleus (VMN) of the hypothalamus, where various neuronal compartments exhibit ultrastructural signs of growth-like activity in response to E_2 treatment [4]. E_2 also exerts growth effects on synapses, such as those seen in the midbrain central gray, where E_2 treatment resulted in an increase in the number of synapses and length of postsynaptic densities [5]. In the VMN [6, 7], hippocampus [8], and amygdala [9], the appearance of dendritic spines is dependent upon variations in the estrous cycle or E_2 treatment itself. The actions of E_2 on synapses and dendritic spines may be indirect. Several studies implicate GABAergic neurons in the hippocampus as mediators of E_2 effects on hippocampal dendritic spines [10, 11]. However, the precise mechanisms by which E_2 exerts its growth-like effects remains to be elucidated. The similarity between E_2 actions and those of brain-derived neurotrophic factor (BDNF) [see reviews in 1, 2] suggest that this neurotrophic factor may be a likely candidate in mediating the effects of E_2 on some of the growth-related processes in neurons and synapses.

Several studies have shown an effect of E_2 on BDNF and/or its message, which appears to vary depending upon the age of the organism, length of ovariectomy, E_2 replacement regimens, and brain area [12–20]. The gene encoding BDNF contains a sequence similar to the canonical E_2 -response element found in E_2 -target genes [12]. Therefore, E_2 actions on BDNF may result from a direct, genomic effect. On the other hand, E_2 may modulate the expression of BDNF as a consequence of activation of the cAMP response element-binding protein (CREB) via protein kinases, such as protein kinase A (PKA), calcium/calmodulin kinase IV (CaMK IV), and mitogen activated protein kinase (MAPK) [21–23]. Phosphorylated CREB (pCREB) can form homo- or heterodimers with cAMP-responsive modulator protein (CREM) or activating transcription factor, and bind to CREB response element (CRE) sites of promoter regions of genes. Together with the appropriate co-activators, the complex can regulate transcription of CREB-related genes, such as BDNF [21–23], proenkephalin [24] and bcl-2 [25], which have growth-like or anti-apoptotic effects in the brain. BDNF can stimulate cellular growth and synaptic remodeling in relevant brain areas and, through these actions, may lead to altered behavioral output. Several reports have shown an effect of E_2 on CREB or pCREB, some of

which indicate rapid, non-genomic actions of this steroid [26–32]. In a series of studies, we found that E_2 treatment to ovariectomized rats increases protein levels of CaMK IV, CREB and pCREB in amygdaloid structures [33, 34]. Immunoperoxidase labeling within the amygdala and hippocampus revealed an increase in pCREB immunoreactivity in subdivisions of the medial amygdala, as well as in CA1 and CA3 regions of the hippocampus [33]. In addition, E_2 decreased immunolabeling for the phosphatase calcineurin in the medial and basomedial, but not central or basolateral, amygdala of ovariectomized rats [35]. Decreased protein levels of calcineurin may allow the persistence of pCREB which, in turn, may stabilize the pCREB/CREB-binding protein complex necessary for efficient CRE-regulated gene transcription [36].

Clinical studies indicate that E_2 may play a role in emotional behaviors [37–42]. We have demonstrated an ameliorative effect of E_2 in the forced swim test, a test for the efficacy of antidepressants [43]. The CREB signaling pathway appears to be a target of antidepressant action [44] and is implicated in synaptic growth and plasticity [45–47]. BDNF has also been implicated as a player in antidepressant action [see for example 48–52 and review in 53], and recent studies have shown that single nucleotide polymorphisms of the *bdnf* gene may be associated with affective [54], as well as cognitive [55] behaviors. However, there are no studies demonstrating an effect of E_2 on both components of the CREB cascade and BDNF protein and mRNA in emotionally-relevant brain areas. We, therefore, investigated the effects of E_2 treatment (10 μ g E_2 for 14 days) to ovariectomized rats on immunolabeling and mRNA levels of BDNF in amygdaloid and hippocampal brain structures. In order to better correlate the changes in BDNF expression with CREB signaling, we also examined the effects of E_2 treatment on levels of CaMK IV, CREB, and pCREB immunolabeling in brain structures of ovariectomized rats.

Materials and Methods

Adult, ovariectomized Sprague-Dawley rats (200–250 g) were purchased from Harlan (Madison, Wisc., USA). All rats were housed 2–3 per cage and adapted to a reverse light/dark cycle (lights on at 20:00 h) for 19 days, which also permitted clearance of endogenous estradiol levels [56]. Rat chow and water were available ad libitum. Animals were tested for completeness of ovariectomy by placing a male rat in a cage with each female for 6 consecutive days to determine the lordosis quotient (number of mounts/number of lordoses) [57]; none of the rats displayed lordosis. Rats were injected subcutaneously with 10 μ g 17 β -estradiol (E_2) in 0.1 ml sesame oil or the vehicle control between 09:00 and 10:00 h for 14

days. Twenty-four hours after the last injection, the animals were perfused with fixative as described below. The injection dosages and protocols chosen were based on published data from our laboratory [see for example, 4, 5, 33–35, 58], as well as those of others [27], so as to optimize the detection of hormonal action on cellular responses and relate our finding to previously published biochemical, molecular, and immunocytochemical data on E₂ effects on the CREB signaling cascade [33–35]. Guidelines for the humane and proper treatment of animals were followed.

Immunocytochemistry Protocol

The protocol has been used previously [35, 59–63] and is briefly described as follows. Rats treated with E₂ or vehicle were deeply anesthetized with Nembutal and transcardially perfused with 100 ml of normal saline, followed by 300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed and postfixed for overnight in the same fixative before being transferred to 10, 20 and 30% sucrose in 0.1 M phosphate-buffered saline (PBS, pH 7.4), at 4°C. The brains were then frozen at –80°C. 20-μm coronal sections were cut at identical bregma levels using a Paxino and Watson [64] atlas as reference through the medial, basomedial, central and basolateral amygdala and through the CA1, CA2, CA3 and hilus and dorsal and ventral dentate gyrus on a cryostat. Sections were processed for immunocytochemistry as described below.

Sections were washed with PBS and blocked for 30 min with RPMI 1640 (Gibco BRL), which we have found to be an effective blocker of non-specific binding for this procedure [61]. This step was followed by 10% normal serum (diluted in PBS containing 25% Triton X-100) for 30 min at room temperature. Sections were then incubated with 1% BSA (prepared in PBS containing 0.25% Triton X-100) for 30 min at room temperature. Sections were further incubated with the primary antibody [CaMK IV (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) (dilution, 1:200); antibodies to CREB and pCREB (Upstate Biotechnology, Waltham, Mass., USA) (dilution, 1:500); antibodies to BDNF (Santa Cruz Biotechnology) (dilution, 1:200)] in 1% BSA prepared in PBS containing 0.25% Triton X-100 for 18 h at room temperature. The dilutions of the antibodies were based on those from previous publications [34, 59–61, 65]. The antibodies used here have been well characterized in our laboratory. Following washes with PBS and 1% BSA in PBS, sections were incubated with 1-nm gold particles conjugated to the appropriate secondary antibody for 1 h at room temperature. For the negative control, the same protocol was used except that 1% BSA in PBS was substituted for the primary antibody. Sections were rinsed several times in 1% BSA, followed by rinses in double-distilled water. Gold particles were then silver-enhanced (Ted Pella, Inc., Redding, Calif., USA) for 12–20 min and washed several times in double-distilled water. Sections were mounted on slides and examined under a light microscope.

Quantification of immunolabeled gold particles was performed with the Loats Image Analysis System connected to a light microscope that calculated the number of gold particles/100 μm² area of defined amygdaloid or hippocampal structures at high magnification (×100), as reported by us previously [34, 35, 59–61]. The threshold of each image was set up in two ways: one within the section itself; areas without labeling gave zero counts and then crossed check with negative sections. Three matching sections for each rat were used for analysis. Three randomly selected fields were chosen for each subnucleus examined of the amygdala or hippocampus.

Calculations were made from these fields from each section. Thus, from one rat, data were pooled from nine fields of three matching sections. For comparisons between E₂-treated and control groups, data was analyzed using the non-parametric Mann-Whitney U-test, as in our previous immunogold studies [34, 35]. A value of $p < 0.05$ was considered significant.

In situ PCR for BDNF mRNA Levels Measurement in Brain

Rat brain sections were used to determine the mRNA levels of BDNF using in situ RT-PCR as described by Pandey et al. [60, 61] and Zhang and Pandey [66]. We used the non-radioactive in situ hybridization methods and the potential to increase the sensitivity is approximately eight times over conventional in situ hybridization methods [67]. 40-μm sections were cut on a cryostat and sections from each of two anterior-posterior [64] levels of interest as described above were processed for in situ RT-PCR as described below.

Sections were washed with PBS and incubated in proteinase K (1 μg/ml in 1 × PBS, 0.05% Triton X-100) for 15 min at 37°C. The reaction was stopped by 0.1 M glycine for 20 min. The sections were washed with 1 × PBS and then subjected to DNase digestion solution (2 μl RNase inhibitor, 8 μl water, 80 μl RQ1 RNase free DNase and 10 μl DNase 10 × buffer I) overnight (12–16 h) at room temperature.

Sections were washed with 1 × PBS and transferred to 100-μl RT reaction mixture containing Oligo d(T) (Applied Biosystems, Foster City, Calif., USA) and reverse transcribed for 30 min at 42°C. For negative control sections, reverse transcriptase was replaced by DEPC-treated water. The sections were transferred to PCR reaction mixtures containing DIG-11-dUTP instead of dUTP. PCR was performed with 0.5 μl *Taq* DNA polymerase enzyme and 1 μl (36–37 pmol) each of BDNF primer. Primers were as follows: forward primer: 5'TAACGGCGGCAGACAAAAGACT3'; backward primer: 5'GTGTCTATCCTTATGAATCGCCAA3'. Following PCR, sections were mounted on slides and BDNF-positive cell bodies were detected by using alkaline phosphate-conjugated anti-digoxigenin antibody and subsequent staining of the complex with specific substrate NBT/BCIP (Roche Molecular Biochemical, Mannheim, Germany). The linearity of the reaction was established first by fixing the RNA and increasing the amplification of the DNA. We used the amplification cycle in the linear range, a procedure we have used in our previous publications [60, 61, 66].

The optical density (OD) of positive areas containing cell bodies was calculated with an image analyzer (Loats Associates). The in situ PCR threshold was set up using negative sections. That is, the OD of negative sections has been subtracted from the OD of the area containing positive cell nuclei. With each amygdaloid or hippocampal subregion analyzed, three randomly selected areas were subject to analysis. The OD of positive cell bodies was calculated by the image analysis system itself, and then values were averaged for each rat. The mRNA results were represented as mean OD/100 pixels area.

Fig. 1. a Photomicrographs of BDNF immunolabeling in amygdaloid structures from ovariectomized (OVX) estrogen (E_2)- and vehicle-treated rats. The top panel shows light micrographs at low magnification of BDNF immunogold labeling in the central amygdala (CeA) and medial amygdala (MeA) from E_2 - and vehicle-treated rats. Scale bar = 400 μ m. Photomicrographs from the CeA (center panel) and MeA (bottom panel) from E_2 - and vehicle-treated rats are shown at higher magnification. Scale bar = 20 μ m.

Results

Effects of Estradiol Benzoate Treatment on BDNF Immunolabeling and Levels of BDNF mRNA Signal in the Amygdala and Hippocampus of Ovariectomized Rats

We used immunogold labeling to determine the localization of BDNF protein within the amygdaloid and hippocampal structures. Immunogold labeling for BDNF was detected in cell bodies and neuronal fibers, as reported previously [60] (fig. 1a). E_2 treatment of ovariectomized rats resulted in an increase in immunogold labeling

in the medial and basomedial, but not central or basolateral, amygdala of ovariectomized rats. Using the central and medial amygdala as representative brain areas, figure 1a shows low and high magnification light micrographs illustrating differences in gold immunolabeling in these two brain regions. Quantification of the data for various structures of the amygdala and hippocampus is shown in the graph in figure 1b. A significant increase in the number of immunogold particles/100 μ m² is seen in the medial (238 ± 5.66 vs. 191 ± 4.83 , $p < 0.01$) and basomedial (227 ± 5.12 vs. 189 ± 6.19 , $p < 0.01$), but not central or basolateral, amygdala of the E_2 -treated group com-

Fig. 1. b Quantitation of BDNF immunogold labeling (number of immunogold particles per 100 μm^2 area) shows that E_2 treatment significantly increases levels of BDNF immunolabeling in specific subdivisions of the amygdala and hippocampus. A significant increase in immunogold labeling is seen in the medial (MeA) ($p < 0.01$) and basomedial (BMA) ($p < 0.01$), but not central (CeA) or basolateral (BLA), amygdala of the E_2 -treated group ($n = 6$) compared to the OVX control animals ($n = 6$). A significant increase in levels of BDNF immunolabeling is seen in the CA1 ($p < 0.001$) and CA3 ($p < 0.001$) regions of the hippocampus, but not in the CA2, dorsal dentate gyrus (DDG), hilus, or ventral dentate gyrus (VDG) of the E_2 -treated group ($n = 6$) compared to the OVX control animals ($n = 6$). Values are the mean $\pm$ SEM. * Significantly different from vehicle-treated rats.

pared to the ovariectomized control animals. Immunogold labeling for BDNF was detected in the cell bodies and neuronal fibres of hippocampal cells (micrograph not shown). A quantitative analysis of the levels of BDNF immunolabeling in subdivisions of the hippocampus is shown in the graph in figure 1b. A significant increase in the immunolabeling (number of gold particles/100 μm^2 of area) is seen in the CA1 (250 ± 8.1 vs. 195 ± 8.03 , $p < 0.001$) and CA3 (261 ± 3.07 vs. 210 ± 6.73 , $p < 0.001$) regions of the hippocampus, but not in the CA2, dorsal dentate gyrus (DDG), hilus, or ventral dentate gyrus (VDG) of the E_2 -treated group compared to the ovariectomized control animals.

To determine the effect of E_2 on mRNA levels of BDNF in the amygdala and hippocampus, we used in situ RT-PCR. The positive cell bodies in the amygdaloid structures showing the signal for levels of BDNF mRNA are shown in figure 2a. E_2 treatment of ovariectomized rats resulted in an increase in the levels of BDNF mRNA in the medial and basomedial, but not central or basolateral, amygdala. These findings are illustrated in figure 2a, which show low and high magnification light micrographs of the medial and central amygdala following E_2 or ve-

hicle treatment to ovariectomized rats as examples. Quantitative analysis of the mRNA levels of BDNF in each of the aforementioned amygdaloid areas is shown in figure 2b. A significant increase in the mean mRNA levels (OD/100 pixels area) is seen in the medial (11.6 ± 0.54 vs. 8.9 ± 0.25 , $p < 0.01$) and basomedial (11.1 ± 0.57 vs. 8.7 ± 0.28 , $p < 0.01$), but not central or basolateral, amygdala of the E_2 -treated compared to the control group.

A quantitative analysis of mRNA levels for BDNF in each of the hippocampal areas is shown in the graph in figure 2b. E_2 administration to ovariectomized rats resulted in an increase in mRNA levels of BDNF in the CA1 and CA3 regions of the hippocampus, but not in the CA2 region or in any of the subdivisions of the dorsal dentate gyrus (DDG), hilus, or ventral dentate gyrus (VDG) of E_2 -treated group compared to ovariectomized controls. A significant increase in the mean levels of signal for BDNF mRNA (OD/100 pixels/area) is seen in the CA1 (12.4 ± 1.15 vs. 8.9 ± 0.88 , $p < 0.001$) and CA3 (14.1 ± 1.16 vs. 10.1 ± 0.79 , $p < 0.001$) regions, but not CA2, DDG, hilus, or VDG of the E_2 -treated group compared to ovariectomized controls.

Fig. 2. a Photomicrographs of BDNF mRNA signal (in situ PCR) in amygdaloid structures from ovariectomized (OVX) estrogen (E₂)- and vehicle-treated rats. The top panel shows light micrographs at low magnification of the levels of mRNA for BDNF in the central amygdala (CeA) and medial amygdala (MeA) from E₂- and vehicle-treated rats. Scale bar = 400 μ m. Photomicrographs from the CeA (middle panel) and MeA (bottom panel) amygdala from E₂- and vehicle-treated rats shows the BDNF mRNA signal at higher magnification. Scale bar = 30 μ m.

Effects of Estradiol Benzoate Treatment on CaMK IV Immunolabeling in the Hippocampus of Ovariectomized Rats

Our previous studies indicated that administration of the E₂ regimen used in the present study had no effect on the immunoreactivity as seen with Western blot analysis of the α -isoform of the PKA catalytic subunit (PKA- α) or levels of PKA activity under basal or cAMP-stimulated conditions [33]. However, this E₂ regimen increases the protein levels of CaMK IV in the nuclear, but not cytoplasmic or membrane fraction of the amygdala of ovariectomized rats [34], as seen with Western blot analysis;

an E₂-induced increase in immunogold labeling was also seen in the medial and basomedial, but not central or basolateral, amygdala of ovariectomized rats [34]. CaMK IV is also a likely candidate for CREB phosphorylation in the hippocampus because CREB is a major substrate for CaMK IV and CaMK IV has been implicated in hippocampal CA1 function [68, 69]. CaMK IV is also the major CREB kinase in activity-dependent neuronal gene expression [36, 70, 71]. In addition, it has been shown that female fertility is reduced in mice lacking CaMK IV [72]. Moreover, there is a growing literature for the role of E₂ in calcium signaling events, including dendritic spine

Fig. 2. b Quantitation of the BDNF mRNA signal shows a significant increase in levels of BDNF mRNA signal in the medial (MeA) ($p < 0.01$) and basomedial (BMA) ($p < 0.01$), but not central (CeA) or basolateral (BLA), amygdala of the E_2 -treated ($n = 5$) compared to the control ($n = 5$) group. A significant increase in levels of BDNF mRNA is seen in the CA1 ($p < 0.001$) and CA3 ($p < 0.001$) regions of the hippocampus, but not CA2, dorsal dentate gyrus (DDG), hilus, or ventral dentate gyrus (VDG) of E_2 -treated group compared to OVX controls. Values are the mean $\pm$ SEM and are represented as the mean OD/100 pixels of area. * Significantly different from vehicle-treated rats.

growth [see discussion in 73]. Also, a significant increase in total CaMK IV protein was seen in the hippocampus following chronic treatment with the antidepressant fluoxetine [74]. We extended the aforementioned CaMK IV studies in the amygdala [34] and examined the effects of E_2 treatment to ovariectomized rats on immunogold labeling of CaMK IV in hippocampal structures. Prior to the present study, there was no data on the effect of E_2 on CaMK IV in the various hippocampal structures.

Figure 3a shows representative areas of brain sections labeled for CaMK IV in the CA1 and CA3 regions of the hippocampus of ovariectomized E_2 - and vehicle-treated animals at high magnification. We found a significant increase in immunogold labeling for CaMK IV (immunogold particles/100 μm^2 area) in the CA1 (98 ± 3.53 vs. 78 ± 3.01 , $p < 0.01$) and CA3 (101 ± 5.62 vs. 78 ± 3.01 , $p < 0.01$) regions of the hippocampus, but not CA2, DDG, hilus or VDG of the E_2 -treated group compared to the control animals (fig. 3b).

Effects of Estradiol Benzoate Treatment on Immunolabeling for CREB and pCREB in the Amygdala and Hippocampus of Ovariectomized Rats

E_2 treatment increased the levels of immunogold labeling for CREB in the medial and basomedial, but not central or basolateral, subdivisions of the amygdala compared to the ovariectomized controls. Figure 4a shows representative low and high magnifications of sections labeled for CREB from the medial and central amygdala of E_2 - and vehicle-treated ovariectomized rats. The graphs in figure 4b show a significant increase in CREB immunogold labeling (number of gold particles/100 μm^2) in the medial (120 ± 4.47 vs. 96 ± 5.47 , $p < 0.001$) and basomedial (114 ± 4.18 vs. 93 ± 3.72 , $p < 0.001$), but not central or basolateral, amygdala of the E_2 -treated group compared to the ovariectomized controls. In contrast, there was no change in CREB immunogold labeling, in any of the subregions of the hippocampus (CA1, CA2, CA3, or dentate gyrus) after 14 days treatment with E_2 (fig. 4b).

A significant E_2 -induced increase in immunogold labeling of pCREB was seen in the medial and basomedial,

Fig. 3. a Photomicrographs of CaMK IV immunolabeling in the hippocampus of ovariectomized (OVX) estrogen (E₂)- and vehicle-treated rats. The top panel shows light micrographs at high magnification of CaMK IV immunogold labeling in the CA1 and CA3 regions of the hippocampus from E₂- and vehicle-treated rats. Scale bar = 30 μ m. **b** Quantitation of CaMK IV immunogold labeling (number of immunogold particles per 100 μ m² area) shows that E₂ treatment significantly increases levels of CaMK IV immunolabeling in specific subdivisions of the hippocampus. A significant increase in immunolabeling is seen in the CA1 ($p < 0.001$) and CA3 ($p < 0.001$) regions of the hippocampus, but not CA2, dorsal dentate gyrus (DDG), hilus or ventral dentate gyrus (VDG) of the E₂- (n = 6) treated group compared to the OVX control animals (n = 6). Values are the mean $\pm$ SEM. * Significantly different from vehicle-treated rats.

but not central or basolateral, amygdala of the E₂-treated group compared to the ovariectomized control animals. Figure 5a shows low and high magnification micrographs of sections labeled with pCREB from the central and medial amygdala from E₂- and vehicle-treated rats as examples (fig. 5a). Quantification of the data indicates a significant increase in the number of gold particles/100 μ m² in the medial (99 ± 3.26 vs. 81 ± 4.58 , $p < 0.001$) and basomedial (102 ± 6.50 vs. 82 ± 4.58 , $p <$

0.01), but not central or basolateral, amygdala of the E₂-treated group compared to the ovariectomized control animals (fig. 5c).

Figure 5b shows representative areas of brain sections labeled for pCREB in the CA1 and CA3 regions of the hippocampus of ovariectomized E₂- and vehicle-treated animals at high magnification. A significant increase in immunogold labeling for pCREB (number of gold particles/100 μ m² area) is seen in the CA1 (112 ± 5.48 vs. 87

Fig. 4. a Photomicrographs of CREB immunolabeling in amygdaloid structures from ovariectomized (OVX) estrogen (E_2)- and vehicle-treated rats. The top panel shows light micrographs at low magnification of CREB immunogold labeling in the central amygdala (CeA) and medial amygdala (MeA) from E_2 - and vehicle-treated rats. Scale bar = 400 μ m. Photomicrographs from the central (center panel) and medial (bottom panel) amygdala from E_2 - and vehicle-treated rats are shown at higher magnification. Scale bar = 30 μ m.

± 3.40 , $p < 0.0001$) and CA3 (120 ± 5.54 vs. 92 ± 3.86 , $p < 0.0001$) regions of the hippocampus, but not CA2, DDG, hilus or VDG of the E_2 -treated group compared to the control animals (fig. 5c). These results are consistent with our previous study using immunoperoxidase (DAB) labeling to demonstrate an E_2 -induced increase in pCREB immunolabeling in the medial amygdala, and CA1 and CA3 regions, but not dentate gyrus, of the hippocampus of ovariectomized rats [33].

Discussion

Our data indicate that the same dose and time regimen of E_2 alters levels of immunolabeling for components of the CREB signaling pathway and the CREB-related gene product BDNF and the levels of mRNA for BDNF. Of particular importance is the observation of neuroanatomical heterogeneity of the E_2 effect within the amygdala and hippocampus. Except for the lack of effect of E_2 on CREB immunolabeling in any of the subregions of the hippocampus, E_2 increased levels of CaMK IV [34], pCREB and BDNF immunolabeling and mRNA levels

Fig. 4. b Quantitation of CREB immunogold labeling (number of immunogold particles per 100 μm^2 area) shows that E_2 treatment significantly increases levels of CREB immunolabeling in specific subdivisions of the amygdala, but not in any of the subdivisions of the hippocampus. A significant increase in the number of gold particles/100 μm^2 is seen in the medial (MeA) ($p < 0.001$) and basomedial (BMA) ($p < 0.001$), but not central (CeA) or basolateral (BLA), amygdala of the E_2 -treated group ($n = 6$) compared to the OVX control ($n = 6$) animals. No differences were seen in the number of gold particles/100 μm^2 in the CA1, CA2, or CA3 regions, or dorsal dentate gyrus (DDG), hilus or ventral dentate gyrus (VDG) of the hippocampus between the E_2 -treated group and OVX control animals. Values are the mean $\pm$ SEM. * Significantly different from vehicle-treated rats.

of BDNF in the medial and basomedial amygdala and CA1 and CA3 regions of the hippocampus. The E_2 -induced increase in pCREB immunolabeling seen in the medial and basomedial subdivisions of the amygdala may be due, in part, to increased CREB protein expression, as well as CREB phosphorylation. In the CA1 and CA3 regions of the hippocampus, on the other hand, the E_2 -induced increase in pCREB immunolabeling may not involve an increase in CREB protein, but only an increase in CREB phosphorylation.

In the present study, levels of pCREB immunolabeling persisted during the 2 weeks of E_2 treatment. The persistence of pCREB immunolabeling may be due to E_2 influences on CaMK IV [34] and/or CREB depending upon the neurocircuitry examined. Alternatively, E_2 may alter levels or activity of phosphatases, such as calcineurin, which regulate CREB phosphorylation. Levels of calcineurin mRNA in the ventromedial hypothalamus are significantly lower during proestrus (when E_2 levels are high) than during diestrus (when E_2 levels are low) [75] and E_2 decreased calcineurin and PP1 activity in the hippocampus [76]. We have recently shown that a similar dose par-

adigm of E_2 decreased calcineurin immunolabeling in the medial and basomedial amygdala and CA1 regions of the hippocampus [35]. These results suggest the possibility that E_2 action on calcineurin may, therefore, contribute to the increased levels of pCREB immunolabeling in the specific neurocircuitry of amygdala and hippocampus of E_2 -treated animals.

Our present studies are consistent with other biochemical and molecular studies showing changes in components of the CREB signaling pathway. Our biochemical data show, using the identical E_2 dose and time regimen as that used in the present study, an E_2 -induced increase in CaMK IV [34], and pCREB and CREB [33] in the amygdala using Western blot analysis. However, in that study, because of the amount of tissue required for the analysis, we were only able to analyze whole amygdala, and not specific subnuclei. Moreover, using the identical E_2 dose and time regimen as that used in the present study, we also demonstrated an E_2 -induced increase in CRE-DNA binding in whole amygdala using electrophoretic gel-mobility shift assay [33]. Furthermore, our present results are consistent with our immunoperoxidase

Fig. 5. a Photomicrographs of pCREB immunolabeling in amygdaloid structures from ovariectomized (OVX) estrogen (E_2)- and vehicle-treated rats. The top panel shows light micrographs at low magnification of pCREB immunogold labeling in the central amygdala (CeA) and medial amygdala (MeA) from E_2 - and vehicle-treated rats. Scale bar = 400 μ m. Photomicrographs from the CeA (center panel) and MeA (bottom panel) amygdala from E_2 - and vehicle-treated rats at higher magnification. Scale bar = 30 μ m.

studies showing an E_2 -induced increase in the number of cells labeled with pCREB in the medial, but not central, amygdala, and CA1 and CA3 regions, but not hilus or dentate gyrus, of the hippocampus [33]. These results indicate that by various biochemical and histochemical approaches, we have achieved results consistent with those using immunogold labeling.

Neuroanatomical Specificity of the E_2 Effect

E_2 -induced alterations in levels of BDNF immunolabeling and mRNA were seen in specific brain structures within the amygdala and hippocampus. In the amygdala,

changes in the parameters examined occurred specifically in the medial and basomedial, but not central or basolateral, amygdala. The amygdala consists of separate nuclei, which may play distinct roles in regulating different aspects of integrated emotional responses [77]. Some areas, such as the medial, central and basolateral amygdala, are associated with fear and anxiety [for discussion, see 78], as well as LTP [78]. In the basolateral amygdala, for example, E_2 has been shown to decrease neuronal excitability [see 78, 79], probably via GABAergic mechanisms [78]. The central amygdala also mediates anxiety and fear behavior [for review, see 80, 81] and aggressive and de-

Fig. 5. b Photomicrographs of pCREB immunolabeling from the CA1 and CA3 regions of the hippocampus from OVX estrogen (E_2)- and vehicle-treated rats at high magnification. Scale bar = 30 μm . **c** Quantitation of pCREB immunogold labeling (number of immunogold particles per 100 μm^2 area) shows that E_2 treatment significantly increases levels of pCREB immunolabeling in specific subdivisions of the amygdala and hippocampus. A significant increase in pCREB immunogold labeling is seen in the medial (MeA) ($p < 0.001$) and basomedial (BMA) ($p < 0.01$), but not central (CeA) or basolateral (BLA), amygdala of the E_2 -treated group compared to the OVX control animals. A significant increase in immunolabeling is seen in the CA1 ($p < 0.0001$) and CA3 ($p < 0.0001$) regions of the hippocampus, but not CA2, dorsal dentate gyrus (DDG), hilus or ventral dentate gyrus (VDG) of the E_2 -treated group ($n = 6$) compared to the OVX control ($n = 6$) animals. Values are the mean $\pm$ SEM. * Significantly different from vehicle-treated rats.

fensive behavior [81]. However, despite the presence of mRNAs for ER α [82] and ER β [83, see also 84 for review] in the central amygdala, mRNA for ER α in the lateral, but not basal, amygdala [see 83 for review], and ER β mRNA in the basolateral amygdala [85, but see also 84], we found no effect on BDNF immunolabeling or mRNA nor immunolabeling for the components of the CREB signaling pathway in these regions with our E₂ regimen in the present study. We cannot, however, rule out a local effect, where E₂ binding in the basolateral and/or central amygdala may have affected the CREB signaling pathway in the medial amygdala. Both ER α and ER β are expressed in the medial amygdala of rat brain [83, 86] and these isoforms are also co-expressed in a large majority of neurons in this region [86]. Others have found that increases in some components of the CREB signaling pathway in some brain regions may not be associated with positive behavior [87–90]; however, those observations depend upon the specific neuroanatomical site, age of the animal, particular behavioral test, and timing of protein expression. Scharfman and MacLusky [2] note that E₂ and BDNF both have similar and dissimilar actions, which can be extensive and complex due to differences in experimental conditions, as well as other as yet unknown variables [2].

In the present study, we found a consistently positive effect on immunolabeling for CREB, pCREB and BDNF and BDNF mRNA in the medial and basomedial nuclei of the amygdala. Previously, we found that E₂ increased the levels of CaMK IV in these nuclei of amygdala [34]. The medial amygdala consists of four subnuclei and has been implicated in a variety of functions, including interpretation of sensory information, social and reproductive behaviors, and the interpretation of emotionally-loaded stimuli [see discussion in 9]. The medial amygdala contains both ER subtypes [83], their specific distribution and quantities varying with the particular medial amygdaloid subnucleus [9]. Frye and Walf [91] have recently shown effects of systemic and/or intra-amygdala (i.e., medial amygdala) E₂, progesterone and/or E₂ plus progesterone on a variety of behaviors relevant to anxiety, fear and pain. Krežel et al. [78] have shown that ER β -deficient female mice display increased anxiety and reduced threshold for the induction of synaptic plasticity in the basolateral amygdala. In addition, there was a concomitant increase in immunolabeling for 5-hydroxytryptamine_{1A} (5-HT_{1A}) in the medial amygdala, suggesting that a local increase in expression of this receptor may contribute to these alterations in the basolateral amygdala [78].

The hippocampus plays a central role in cognition and affect and CREB signaling may also be involved. Some of the functional consequences of E₂, CREB signaling and increased BDNF impact dendritic spine growth and plasticity in the CA1 region of the hippocampus [for reviews, see 1, 2]. In terms of structural effects, components of the CREB signaling pathway, including CaMK IV, have been implicated in synaptic growth and/or remodeling [45–47]. In terms of function, Marie et al. [47] showed that acute expression of active CaMK IV or CREB in CA1 pyramidal cells resulted in an enhancement of NMDA-receptor-mediated synaptic responses and LTP. These data were coincident with electrophysiological and structural alterations in accordance with the generation of ‘silent synapses,’ which permit additional experience-dependent changes in neural circuits. These adaptations may be necessary for the consolidation of long-term synaptic plasticity and memories.

Effects of E₂ on Levels of BDNF Immunolabeling and Levels of mRNA for BDNF

The effects of E₂ on levels of BDNF mRNA are well documented [12–20]. However, disparities in hippocampal or amygdaloid responses to E₂ may be due to differences in E₂ regimens (acute or chronic), modes of delivery (injections vs. pellets), length of time ovariectomized (acute vs. chronic), age of the animal when E₂ replacement begins (young adult vs. old) and organism (strain of rats; rats vs. voles). Even in intact, cycling rats, variations in levels of BDNF mRNA were seen in different hippocampal areas during proestrus [see 14, 19], which may be attributed to different times of sacrifice during that phase [19]. Scharfman et al. [20] found an increase in BDNF immunoreactivity on the day of proestrus and on the morning of estrus, compared to that seen on the day of metestrus or in ovariectomized rats. Although the highest concentration of BDNF was seen in the mossy fiber axons of dentate gyrus granule cells, increased immunoreactivity was also seen in neuropil containing dendrites of CA1 and CA3 neurons. The estrus cycle changes in BDNF corresponded to alterations in neurophysiological responses in CA1 and CA3 neurons to Schaffer collateral stimulation or repetitive hilar stimuli, respectively; slices taken from ovariectomized animals showed no hyperexcitability in the CA3 region. Cavus and Duman [19] found no differences in levels of BDNF mRNA in the CA1 and CA3 regions following chronic E₂ treatment (E₂ pellet delivery for 2 weeks) of ovariectomized rats. In our study, we observed an E₂-induced increase in BDNF immunolabeling and levels of mRNA for BDNF in those

regions. This discrepancy may be due to the length of time ovariectomized prior to hormonal treatment, i.e., less than a month in the present study vs. 3 months in the study of Cavus and Duman [19]. These differences are consistent with the notion that E₂ replacement is effective in altering BDNF mRNA levels if given soon after ovariectomy, but become refractive if given after prolonged E₂ deprivation [13, 92]. Whether or not the responsiveness of the *bdnf* gene to E₂ is an issue is unknown at present because the observed E₂ effects on BDNF mRNA may be direct, possibly via an ERE in the *bdnf* gene [93, see also 94] or indirect via CREB signaling.

Significance

The percent of E₂-induced increases in protein levels of CREB, pCREB, BDNF and mRNA levels of BDNF mRNA in the medial amygdala range from about 24 to 30% and in the hippocampus, from about 24 to 39% (except for hippocampal CREB levels themselves). That alterations of this magnitude in the levels of pCREB can alter behavior is evidenced from studies from Pandey et al. [61], who showed that using alcohol-withdrawn (24 h) rats following chronic alcohol administration (15 days) or pharmacological manipulations of CREB phosphorylation, a 32% decrease in pCREB resulted in a significant reduction in open arm activity (entries and percent time spent on open arms) in the elevated plus maze. In addition, about 40% reductions in CREB and pCREB protein levels are associated with 40% reductions in the protein levels of BDNF in the amygdala and hippocampal structures of CREB haplodeficient mice and behaviorally these mice displayed depression and anxiety-like behaviors [60].

Since CREB along with BDNF function has been implicated in cognition, depression and anxiety-like behaviors [62, 95–97, see also 98], it is possible that ability of E₂ to increase CREB and BDNF function in selected circuitry of the amygdala and hippocampus may related to its positive effects on the same type of behaviors. Thus, the CREB signaling cascade represents a potential pathway for E₂'s neuroprotective effects. Also, growth effects of E₂ have been well documented [for reviews, see 1, 2] and many of these actions may occur via increased BDNF or other neurotrophic factors. The resultant increased survival of neural circuits and/or strengthening of neural connectivity may counteract some of the deleterious effects of stress on the brain that may ultimately lead to depression [44; see discussion in 33]. Nibuya et al. [99] have shown that the downregulation of BDNF mRNA in response to stress is inhibited by long-term antidepressant treatment. BDNF infused into the midbrain [48] or

hippocampus [49] has been shown to have antidepressant properties in the forced swim test, a test for the efficacy of antidepressants in rodents [100]. Overexpression of CREB in the hippocampus also alters behavior in the forced swim test [22]. We have shown an ameliorative effect of E₂, albeit with a shorter regimen (10 µg E₂ for 7 days), in the forced swim test [58]. The trophic effects of E₂, acting either by genomic mechanisms or non-genomic mechanisms, such as have reported in the present study, may raise the threshold for stress-related neuronal damage. These molecular pathways may also be part of the normal repertoire for several steroid-sensitive neuronal circuits [1].

Our data identify a signaling pathway through which E₂ increases the expression of BDNF that may underlie some of the effects of E₂ on affective and cognitive function. Our immunogold experiments localizing the E₂ effect on components of this pathway to the medial and basomedial amygdala and CA1 and/or CA3 hippocampus. Moreover, our results indicate the heterogeneity of the E₂ effect in the amygdala and hippocampus. Chronic administration of some antidepressants increased CREB-mediated gene transcription and phosphorylation of CREB in specific subdivisions of the amygdala [101] and hippocampus [101, 102]. That E₂ can regulate various components of the CREB signaling and BDNF in specific limbic areas suggests an ability of this hormone to modulate subtleties of neuronal function that are involved in complex behaviors and individual differences associated with them.

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