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ORIGINAL PAPER

Estrogenic regulation of gene and protein expression within the amygdala of female mice

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Abstract Estrogens exert important actions on fear and anxiety in both humans and non-humans. Currently, the mechanisms underlying estrogenic modulation of fear are not known. However, evidence suggests that estrogens may exert their influence on fear and anxiety within the amygdala. The purpose of the present study was to examine the genomic effects of estrogens within the amygdala of female mice using high-density oligonucleotide microarrays. We examined the effects of estrogens on gene expression at 2 and 24 h after an acute subcutaneous injection. Data from the microarrays revealed that 2 h following an acute injection of estradiol, 44 genes were significantly up- or downregulated, and at 24 h, 13 transcripts were significantly up- or downregulated. One interesting estrogen-regulated gene, (CaMKIIα), was downregulated ninefold 2 h following an acute estradiol injection but was not altered 24 h after injection. We further examined estrogen regulation of CaMKIIα, as well as CaMKIIβ and CaMKIV within the amygdala using quantitative PCR and western blot analysis. The data indicate that estrogen decreases CaMKIIα and CaMKIV but not CaMKIIβ gene expression within the amygdala. However, CaMKII protein levels were not different, and CaMKIV protein levels increased 2 h post-EB treatment. These results indicate that estrogen regulates CaMK gene expression and protein levels within the amygdala.

Keywords Quatitative PCR · Microarray · Anxiety · Fear

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Introduction

It is well known that estrogens affect mood and psychiatric disorders including depression and anxiety. Twice as many women as men are affected by depression, and significantly more women than men suffer from a variety of anxiety disorders [1, 2]. The exact cause of this gender difference is unknown. However, a likely basis for these differences may be attributed to a class of steroid hormones, the estrogens. Evidence suggests that changes in circulating levels of estrogens are linked to alterations in mood and emotion as well as to the development of mental illness in females [3–6]. Although a link has been made between estrogens and emotion, the neurobiological mechanisms underlying this connection have remained elusive.

There are several mechanisms, which characterize the actions of estrogens within the central nervous system. Estrogen signals can be transduced through the activation of nuclear estrogen receptors, which specifically bind to DNA at estrogen-responsive elements and regulate gene transcription, and, ultimately protein synthesis. Estrogens can also act on the components of second messenger pathways and additional nuclear proteins to indirectly affect gene transcription [7–10]. In addition, estrogens can have non-genomic effects that include rapid actions on neuronal excitability, activation of cyclic AMP and MAPK pathways, and alterations in calcium channels and calcium

ion entry [11–14]. Within hippocampal slices, estrogens regulate synaptic plasticity, in part, through the activation of the MAPK signaling pathway. Interestingly, the early membrane effects of estrogen can amplify the slower genomic actions of the steroid hormone [15], suggesting that the two pathways can act synergistically. Taken together, these data suggest that estrogens can have a multitude of effects on neuronal function which can then have a profound impact on behavior.

The amygdala and its afferent and efferent projections are important for the acquisition and expression of conditioned fear [16, 17]. Whereas the neural circuitry underlying aversive learning in mammals has been well characterized, relatively little is known about the actions of estrogens within the amygdala and how they may modulate emotion. Both α-estrogen receptor (ERα) and β-estrogen receptor (ERβ) genes are widely distributed throughout this region in both rodents and humans [18–22], suggesting that the amygdala is a likely site at which estrogens exert some of their actions on aversive learning as well as on other emotions.

Therefore, the present study aims to begin parsing the molecular actions of estrogens within the amygdala, in order to gain a more complete understanding of how estrogens modulate amygdala function.

Materials and methods

Animals and housing

For all experiments, adult female ovariectomized, Swiss–Webster mice (*Mus musculus*) were obtained from Taconic Farms. Animals were group-housed (five animals per cage) 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 Plexiglass cages (26.5 × 16 × 20 cm^3) with corn cob (Bed-O-Cob) bedding, with wire mesh tops, 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.

Experiment 1: effects of estrogen on gene expression in the amygdala

Forty 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 then matched by weight and assigned to one of four experimental conditions. Animals in Group 1 were administered a single subcutaneous (s.c.) injection of estradiol benzoate (EB) (10 μg in 0.1 ml sesame oil), whereas animals in Group 2 were administered a single s.c. injection of 0.1 ml vehicle control (sesame oil). This concentration of EB has previously been used by our lab to investigate non-reproductive behaviors in mice and produces a proestrus level of circulating estrogen [23, 24]. Mice in Groups 1 and 2 were killed 2 h following injections. Animals in Group 3 were administered a single s.c. injection of EB (10 μg in 0.1 ml sesame oil), whereas animals in Group 4 were administered a single s.c. injection of 0.1 ml vehicle control (sesame oil). These animals were then killed 24 h following injections. The brains of all animals were removed immediately at the designated time, placed in an adult mouse brain matrix (Ted Pella, Inc., Redding, CA), chilled on ice, and sectioned into 1 mm coronal slices. The amygdala was dissected bilaterally from the coronal slices by making two cuts, one beginning immediately lateral to the optic tract and rising dorsally 1.5 mm and the other laterally through the temporal lobe. This dissection included the entire amygdala complex as well as the piriform cortex and a portion of the insular cortex from bregma −1.06 through bregma −2.06 (Fig. 1, left). The dissected tissue was placed in ice-cold RNAlater® (Ambion, Austin, TX) and immediately frozen and stored at −80°C until processing. 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 RNA from animals within each group was pooled and was then used to construct cRNA for hybridization to the GeneChip®. The Murine Genome U74v2 GeneChip® arrays were purchased from Affymetrix. For each pooled sample, 5 μg of poly(a)$^+$ RNA was converted to biotinylated cRNA according to the Affymetrix protocol and hybridized to a single GeneChip®. The hybridization, washing, and scanning were carried out at The Rockefeller University Gene Array Resource Center.

Experiment 2: effects of estrogen on CaMK gene expression in the amygdala: quantitative PCR

Based on the data obtained from the microarray experiment, we further investigated the effect of estrogen on gene expression within the amygdala using quantitative real-time PCR. Because of its involvement in fear learning we chose to further examine calcium/calmodulin-dependent protein kinase II alpha (CaMKIIα) gene expression by estrogens. Specifically, CaMKIIα was demonstrated to be decreased by ninefold 2 h following estrogen treatment but back to normal levels 24 h later. In order to more accurately determine the estrogen's effect on gene expression in

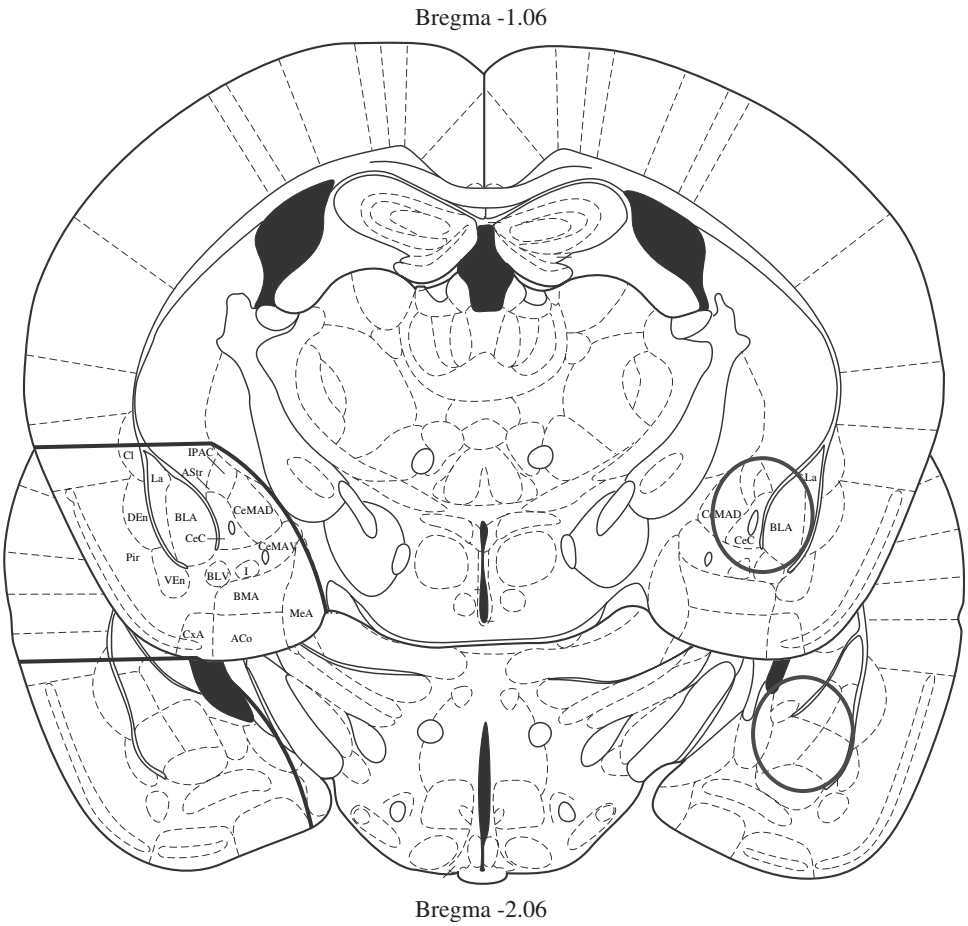

Fig. 1 Coronal sections of the mouse brain illustrating the region and the rostro-caudal extend of the amygdala dissected for Experiment 1 (microarray, left) and Experiments 2 and 3 (qPCR and protein, right). Drawings are adapted from Paxinos and Franklin (2001). La, lateral amygdala; BLA, basolateral amygdala; BLV, basolateral amygdala (ventral); CeC, central amygdala (capsular); CeMAD, central amygdala (medial aterodorsal); CeMAV, central amygdala (medial ateroventral); BMA, basomedial amygdala; MeA, medial amygdala; ACo, anterocortical amygdala; CxA, cortex-amygdala transition zone; Pir, piriform cortex; I, intercalated nucleus of the amygdala; Astr, amygdalostriatal transition area; Cl, claustrum; Den, dorsal endopiriform cortex; VEn, ventral endopiriform cortex

the amygdala, we investigated estrogen's regulation of CaMKIIα as well as CaMKIIβ and CaMKIV within the amygdala using quantitative real-time PCR and a more precise dissection technique. In this experiment, adult female ovariectomized Swiss–Webster mice ($n = 24$) were matched by weight and assigned to one of four groups. The first group was administered a single s.c. injection of EB (10 μg), whereas Group 2 was administered vehicle control (sesame oil). Mice in Groups 1 and 2 were killed 2 h following injections. The third group also received a single s.c. injection of EB (10 μg), whereas Group 4 was administered vehicle control. Mice in Groups 3 and 4 were killed 24 h following injections. In all groups, brains were rapidly removed and dissected into ice-cold Artificial Cerobro-Spinal Fluid (ACSF). The brains were then blocked and adhered to the chuck of a vibratome with cyanoacrylate glue and sliced in ice-cold sucrose-ACSF to the amygdala corresponding to 1.06 mm posterior to bregma (Fig. 1, right). A 1.0 mm slice was then taken and a 1-mm diameter area encompassing the lateral, basolateral, and central nucleus of the amygdala was dissected out bilaterally using a stainless steel punch tool (Fig. 1, right), deposited into separate 1.5 ml microcentrifuge tubes containing ice-cold RNAlater® and then immediately 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, 200 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 200 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, 1:30 cDNA: H_2O and stored at −20°C until assayed.

Absolute quantitation using a standard curve method was used to quantify cDNA levels during quantitative PCR

Table 1 Forward and reverse primers and GenBank accession numbers for each gene analyzed using qPCR

Gene	GenBank	Forward primer	Reverse primer
CaMKIIα	NM_177407	5′-AAACTTGTGGTCCCGGAACA-3′	5′-TGCGGATATAGGCGATGCA-3′
CaMKIIβ	NM_007595	5′-GTAAACATCTGCCAGGCTCCA-3′	5′-CCCAAATTATCCCAGACAGGC-3′
CaMKIV	NM_009793	5′-TCAGCAGGTCAAGCACAAACC-3′	5′-ATCACCCTCGTCAGATGATGC-3′

according to [25, 26]. In order to construct standard curves, mouse cDNA was PCR amplified using the same primers, which yielded a single fragment of 101, 171, and 102 bp for CaMKIIα, CaMKIIβ, and CaMKIV, respectively. PCR fragments were 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 clones were re-analyzed for size and presence of unique restrictions sites and then linearized. A standard calibration curve was then prepared for each gene in which the cloned sequence was present at 3.0×10^5–3.0×10^1 copies and then stored at −80°C until assayed. Quantitative PCR was conducted on an ABI Prism 7000 Sequence Detection System (Applied Biosystems) in 20 µl volumes using SYBR green dye on the experimental samples and the standard calibration curve, in triplicate on a single 96-well plate for each group. The primers for quantitative PCR were designed using PRIMER EXPRESS® software (Applied Biosystems) (Table 1), and the appropriate final concentrations were determined empirically.

Experiment 3: effects of estrogen on CaMK protein levels in the amygdala

In this experiment, adult female ovariectomized Swiss–Webster mice ($n = 21$) were matched by weight and assigned to one of four groups, and treated as described in Experiment 2. In all groups, brains were rapidly removed, quickly frozen on dry ice, and stored at −80°C until processing. Amygdala punches were obtained by mounting the brain to the chuck of a cryostat, and then sliced to the amygdala corresponding to 1.06 mm posterior to bregma (Fig. 1, right). A 1-mm diameter area encompassing the lateral, basolateral, and central nucleus of the amygdala was dissected out bilaterally using a stainless steel punch tool, to a depth of 1 mm, and deposited into separate 1.5 ml microcentrifuge tubes and then immediately stored at −80°C until processing.

Amygdala tissue was lysed with sodium dodecyl sulfate (SDS) lysis buffer containing 1% SDS, 50 mM Tris–HCl (pH 7.5), 5 mM EDTA, 10 mM NaF, 2 mM Na_3VO_4, 2 mM $MgCl_2$, 150 mM NaCl, 2 mM $Na_4P_2O_7$, protease inhibitor (Roche; 200 µl), boiled for 5 min and assayed for protein content by the BCA protein Assay Kit (Pierce Chemical). Aliquots of protein (15 µg) were separated by electrophoresis through SDS–PAGE gel and blotted to a nitrocellulose membrane. The membranes were blocked overnight at 4°C with 0.1% Tween20/TBS containing 5% non-fat dried milk. Membranes were then probed with the following antibodies: anti-CaMKIIα, anti-CaMKIIβ (Promega, Madison, WI) anti-CaMKIV (BD Biosciences, San Jose, CA) and anti-β-actin (Promega, Madison, WI) (1:5,000 for all antibodies). The immunoreactive deposits were detected by enhanced chemiluminescence according to the manufacturer's instructions (Limiglo; Cell Signaling, Charlottesville, VA). Relative optical density (ROD) of western blots was measured using the image analysis software (MCID, Imaging Research Inc, St. Catherines, ON, Canada). The changes in protein levels were expressed as a ratio to β-actin.

Statistical analysis

For Experiment 1, the data were analyzed using MICRO-ARRAY SUITE software (Affymetrix). A comparison analysis was made between two samples on two different GeneChips® (i.e., comparisons were made between vehicle-treated and EB-treated tissue, with vehicle serving as the baseline measurement). This analysis compares the different values of each probe pair in the baseline array (vehicle-treated) to its matching probe pair on the experimental array (EB-treated). A quantitative assessment of the change in gene expression is reported as the "signal log ratio" which is then converted to fold-change. A fold-change of at least ±2 was used as a cut-off to determine whether genes were significantly different. For Experiment 2, the data were analyzed using a Mann–Whitney U -test, and in Experiment 3, data were analyzed using a Student's t-test. For all statistical analyses, significance was ascribed at $P < 0.05$.

Results

Estrogen treatment regulates CaMK mRNA expression in the amygdala: affymetrix microarray

Microarrays hybridized with RNA from the amygdala of EB-treated and vehicle-treated mice revealed that a total of

44 genes were regulated at least ±2-fold 2 h after EB treatment, and 13 genes were regulated at least ±2-fold 24 h after EB treatment. Table 2 lists genes regulated by estrogen. Specifically, 2 h after EB treatment, ten genes were upregulated, whereas 34 genes were downregulated by at least twofold. Twenty-four hours after the EB treatment, 2 genes were upregulated, whereas 11 genes were downregulated (Table 2). Transcripts that were regulated 2 h post-EB injection were not the same genes regulated at 24 h after injection. One of the more interesting genes regulated by EB was CaMKIIα, a Ca^{2+}-activated kinase that is highly abundant in the brain and is involved in learning and memory, including emotional memory [27–29]. Results from the microarray scan indicated that EB treatment induced a ninefold decrease in CaMKIIα gene expression 2 h after EB treatment but not after 24 h. Because of the relatively large dissection area of the tissue used for the microarrays, we sought to confirm this finding using a more precise dissection of the amygdala as well as using an additional technique (quantitative PCR). In addition, we also examined the related Ca^{2+}-activated genes, CaMKIIβ and CaMKIV.

Estrogen treatment regulates CaMK mRNA expression in the amygdala: quantitative PCR

The standard curve for CaMKIIα, CaMKIIβ, and CaMKIV was $r^2 = 0.999$ for each gene. The qPCR runs yielded a single product as analyzed by a melting curve and by gel electrophoresis. In addition, qPCR on the no-RT control samples did not amplify any product demonstrating the absence of genomic DNA contamination. There was a significant effect of EB treatment on CaMKIIα and CaMKIV gene expression after 2 h ($P < 0.05$) (Fig. 2), but no significant effect on CaMKIIβ gene transcription, although there was a trend approaching significance for this transcript ($P = 0.07$) (Fig. 2a). Specifically, EB treatment decreased CaMKIIα and CaMKIV gene expression compared with vehicle treatment ($P < 0.05$). Twenty-four hours after the EB treatment, there was no significant effect on CaMKIIα and CaMKIIβ gene expression ($P > 0.05$) (Fig. 2b), an effect that was similar to the pattern observed using the affymetrix microarrays (CaMKIIα). However, at 24 h, CaMKIV gene expression was decreased compared with vehicle treatment ($P < 0.05$) (Fig. 2b).

Estrogen regulation of CaMK protein in the amygdala

The western blot analysis indicated that EB treatment did not significantly regulate CaMKII protein expression. Specifically, there were no differences in CaMKIIα, CaMKIIβ protein levels within the amygdala between vehicle-treated and EB-treated mice at either 2 or 24 h after injection ($P > 0.05$) (Fig. 3a, b). However, there was a significant increase in CaMKIV protein within the amygdala 2 h after EB treatment compared with vehicle-treated mice ($t_7 = 2.394$; $P < 0.05$) (Fig. 3a). There was no difference in CaMKIV protein levels in the amygdala 24 h later in EB-treated mice compared with vehicle-treated mice ($P > 0.05$, Fig. 3b).

Discussion

These data demonstrate that estrogens regulate a variety of gene transcripts within the amygdala. It is interesting to note that the transcripts regulated at 2 h post-EB injection were not the same transcripts observed to be regulated 24 h after injection and suggesting differential effects of the fast versus the slow genomic actions of ER activation. In addition, vasopressin mRNA was observed to be upregulated 4.5-fold 24 h after EB treatment but not 2 h after injection, confirming previous reports demonstrating that vasopressin expression is regulated by estrogens and ERs [30, 31].

The present data suggest that CaMKIIα and CaMKIV transcript levels are regulated by estrogen within the amygdala. These data are functionally significant given that both of these gene transcripts are involved in emotional learning [29, 32–35] and because of estrogen's known effects on emotional learning [23, 24, 36, 37]. It is important to note that there was a difference in the degree of regulation as measured by the affymetrix microarray compared with qPCR. The microarray indicated a ninefold decrease in CaMKIIα expression 2 h after an acute injection of EB. However, the qPCR assay indicated a modest decrease in transcript levels of approximately 40%. This is most likely due to the differences in the dissection of the amygdala region, with a more precise dissection of the amygdala in the qPCR experiment (see Fig. 1). This might indicate that much of the differences in CaMKIIα transcript levels observed with the microarrays was due to the changes taking place outside of the dissected region, possibly in the medial or anterior cortical amygdala, two regions within the larger dissection area containing ERs [18].

A recent study reported a long-term estrogen treatment (2 weeks) to increase CaMKIV protein expression in the medial amygdala but not in the central or basolateral nuclei of the amygdala of rats [38]. In the present study, we found EB treatment to decrease both CaMKIIα and CaMKIV mRNA expression in tissue dissected mainly from the lateral, basolateral, and central nuclei of the amygdala. However, we did observe an increase in CaMKIV protein

Table 2 Transcripts up- or downregulated within the amygdala 2 and 24 h after an acute injection of EB

Probe set ID	Gene title	Gene symbol	Fold change	GenBank
Genes regulated 2 h post-EB injection				
100956_at	Klotho	Kl	−11.96	NM_013823 [18]
101553_at	Fibrinogen, alpha polypeptide	Fga	−3.58	NM_010196 [4]
102559_at	Bone morphogenetic protein 2	Bmp2	−3.56	AK133923 [18], NM_007553 [18]
102893_at	POU domain, class 2, transcription factor 1	Pou2f1	−2.17	AK082573 [18], NM_011137 [18], NM_198933 [18]
103460_at	DNA-damage-inducible transcript 4	Ddit4	−2.14	NM_029083 [18]
104158_at	SNW domain containing 1	Snw1	−2.14	NM_025507 [23]
104328_at	Aquaporin 9	Aqp9	−3.53	AK154151 [13], BC024105 [13], NM_022026 [18]
160060_at	Zinc finger, HIT type 3	Znhit3	−5.82	BC089554, NM_001005223
160417_at	Kinesin family member 5B	Kif5b	2.06	AK014590 [18], NM_008448 [18]
160583_at	Extra cellular link domain-containing 1	Xlkd1	−6.45	NM_053247 [13]
160700_i_at	Sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6C	Sema6c	−3.46	BC059007 [13]
160721_at	E74-like factor 1	Elf1	−10.06	NM_007920 [18]
160918_at	S100 calcium binding protein G	S100g	−3.27	BC010751 [13], NM_009789 [13]
161191_i_at	G-rich RNA sequence binding factor 1	Grsf1	−2.68	AK208573, NM_178700
161453_r_at	G-rich RNA sequence binding factor 1	Grsf1	6.32	AK208573, NM_178700
161866_at	Eph receptor A4	Epha4	−3.32	NM_007936
162046_at	5′-nucleotidase, cytosolic III-like	Nt5c3l	−5.54	AV377022
162068_r_at	Embigin	Emb	−2.14	AK148540, NM_010330
162187_f_at	Regenerating islet-derived 3 gamma	Reg3g	−2.08	BC046602, NM_011260
92402_at	Similar to putative retrovirus-related gag protein	LOC666623	−5.43	XM_985034 [27]
92601_at	Pancreatic lipase related protein 1	Pnliprp1	−3.58	BC068266 [23], BC090985 [18], NM_018874 [18]
93294_at	Connective tissue growth factor	Ctgf	2.60	NM_010217 [18]
93376_at	BMP2 inducible kinase	Bmp2k	4.17	AK046752 [13]
93441_at	RIKEN cDNA 2700099C18 gene	2700099C18Rik	−9.45	AK132724 [13], BC002320 [13], XR_001643 [23], XR_004581 [23], XR_005146 [23]
93640_s_at	Testis expressed gene 21	Tex21	10.27	AK016434 [13], BC100432 [18], NM_019784 [18], XM_001001879 [18], XM_988971 [18], XM_989009 [18]
93660_at	Calcium/calmodulin-dependent protein kinase II alpha	Camk2a	−9.13	NM_009792 [18], NM_177407 [13]
94090_at	T lymphoma oncogene	Tlm	−4.82	NM_011601 [28]
94684_at	Density-regulated protein	Denr	−6.45	AA517134, AA516942
95350_at	Transthyretin	Ttr	−41.93	NM_013697 [18]
96216_at	ORM1-like 1 (*Saccharomyces cerevisiae*)	Ormdl1	5.28	AK050385 [18], NM_145517 [18]
96353_at	Transmembrane protein 14C	Tmem14c	2.03	AK089021 [29], BC069899 [18], NM_025387 [18]
97197_r_at	Expressed sequence AI506816	AI506816	2.07	XM_001000690 [27], XM_001001378 [29]
97216_at	Pregnancy zone protein	Pzp	−2.73	NM_007376 [18]
97438_r_at	Ankyrin repeat and IBR domain containing 1	Ankib1	2.00	NM_001003909 [18]
97654_at	Distal-less homeobox 6, antisense	Dlx6as	−3.63	NR_002839 [18]
97739_at	Ubiquitin-conjugating enzyme E2O	Ube2o	−2.00	NM_173755 [18]
98054_at	Extracellular matrix protein 1	Ecm1	−4.66	NM_007899
98247_at	Hypothetical protein LOC637741	LOC637741	−2.25	XM_914838 [28]
99011_at	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3	Galnt3	−2.75	NM_015736 [23]
99032_at	RAS, dexamethasone-induced 1	Rasd1	2.64	NM_009026 [18]

Table 2 continued

Probe set ID	Gene title	Gene symbol	Fold change	GenBank
99163_at	RIKEN cDNA 4921506J03 gene	4921506J03Rik	−7.41	AK014824 [18], BC079579 [18]
99237_at	cDNA sequence U55872	–	−3.94	U55872(18)
99840_at	Prodynorphin	Pdyn	−4.44	NM_018863 [18]
99905_at	Deubiquitinating enzyme 2	Dub2	−4.69	NM_001001559 [16]
Genes regulated 24 h Post EB Injection				
103539_at	Cytoplasmic tyrosine kinase, Dscr28C related (Drosophila)	Tec	−4.50	NM_013689 [18]
160342_r_at	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 2	Ndufb2	−2.31	AK028334, BC013510, NM_026612
160711_at	2,4-dienoyl CoA reductase 1, mitochondrial	Decr1	−2.11	NM_026172 [18]
161646_r_at	Kelch domain containing 2	Klhdc2	6.42	AK217098, NM_027117
161806_r_at	ATPase, H+ transporting, lysosomal accessory protein 1	Atp6ap1	−2.07	AK180096, NM_018794,XM_972661
162190_r_at	Limb region 1 like	Lmbr1l	−2.04	AK173121, NM_029098
93166_at	PREDICTED: *Mus musculus* hypothetical protein LOC626765 (LOC626765), mRNA	–	−12.38	AI662429, AA183920, AA104741,AI591865
93381_at	Arginine vasopressin	Avp	4.54	AK138179 [18], NM_009732 [18]
94061_at	Cysteine-rich protein 1 (intestinal)	Crip1	−4.17	BC030406 [29], BC064074 [18], NM_007763 [18]
94331_at	Signal transducer and activator of transcription 6	Stat6	−4.63	AK149563 [27],BC029318 [18]
94773_at	Kallikrein 1-related pepidase b4	Klk1b4	−2.20	NM_010915 [18]
94782_at	Expressed sequence C78142	C78142	−9.71	C78142
96309_r_at	RIKEN cDNA 1100001I22 gene	1100001I22Rik	−2.35	BC086936, NM_026724

levels but not CaMKIIα or CaMKIIβ protein expression in the same region. These data seem to contradict the findings of Zhou et al. [38] in that CaMKIV protein expression was increased within the central and basolateral amygdala. The differences in estrogen treatment between the two studies, however, may explain the differences in the results. In the present study, mice were given a single acute injection of EB, whereas in the study conducted by Zhou et al., animals received daily injections for 2 weeks. It is possible that compensatory mechanisms masked a fast increase in CaMKIV protein within the lateral and basolateral amygdala in their study. For instance, in the present study, CaMKIV protein levels were returned to baseline levels within 24 h of EB treatment. Interestingly, these data suggest that although CaMK transcripts decrease as measured by microarray and qPCR these changes did not translate into decreased protein levels, and for CaMKIV, resulted in increased protein levels. The mechanism underlying the differential response remains unknown, but suggests differential roles of estrogens in transcription and translation. Future experiments will need to examine changes in phosphorylated levels of CaMK's in response to EB treatment in order to better understand how estrogens regulate CaMK activity within the amygdala.

As mentioned above, estrogens can have profound effects on fear and anxiety-like behavior. EB-treated mice display increased retention of a passive avoidance task [36] and increased freezing to a contextual and conditioned stimulus [23, 24], indicating a facilitation of fear and anxiety-like behavior (contextual fear). Estrogen's facilitation of both contextual and cued fear as well as instrumental learning is consistent with its known effects on the hippocampus, the hypothalamus, and more recently the amygdala. Likewise, it was demonstrated that corticotropin-releasing hormone (CRH) gene expression within the central nucleus of the amygdala was upregulated by EB treatment, providing a mechanism through which estrogens, in part, modulate fear and anxiety in females [23].

Estrogen may regulate CaMKIIα, CaMKIV, and CRH by either binding to one or both of the ERs in the amygdala or through second messenger pathways and additional transcription factors to indirectly affect gene transcription [7, 8, 10]. One question that remains unanswered is which cell types within the amygdala estrogen acts upon to decrease CaMK gene expression. There is evidence that ERs are expressed in parvalbumin-containing inhibitory neurons within the cortex, hippocampus, basal forebrain as well as the amygdala [39], suggesting that estrogen can

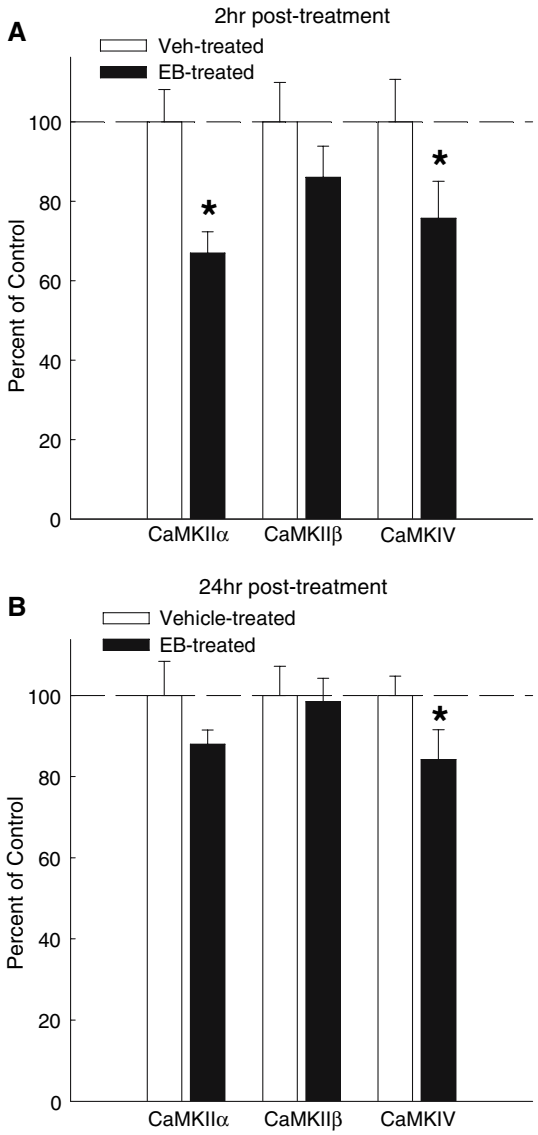

Fig. 2 Mean (±SEM) of CaMKIIα, CaMKII*β*, and CaMKIV mRNA levels in the amygdala from EB-treated and vehicle-treated mice killed 2 h (**a**), and 24 h (**b**) after treatment. 200 ng of total RNA from the amygdala was analyzed by quantitative real-time PCR using the primers and reaction conditions as described above. mRNA copy numbers were calculated from the standard curve of each gene generated simultaneously. Copy number was then transformed into percentage of control (vehicle-treated mice) per 200 ng of total RNA. EB treatment significantly decreased CaMKIIα and CaMKIV, but not CaMKII*β* gene expression in the amygdala 2 h after treatment. Twenty-four hours after EB treatment, only CaMKIV gene expression was reduced in the amygdala. Significant differences are denoted by an asterisk (*)

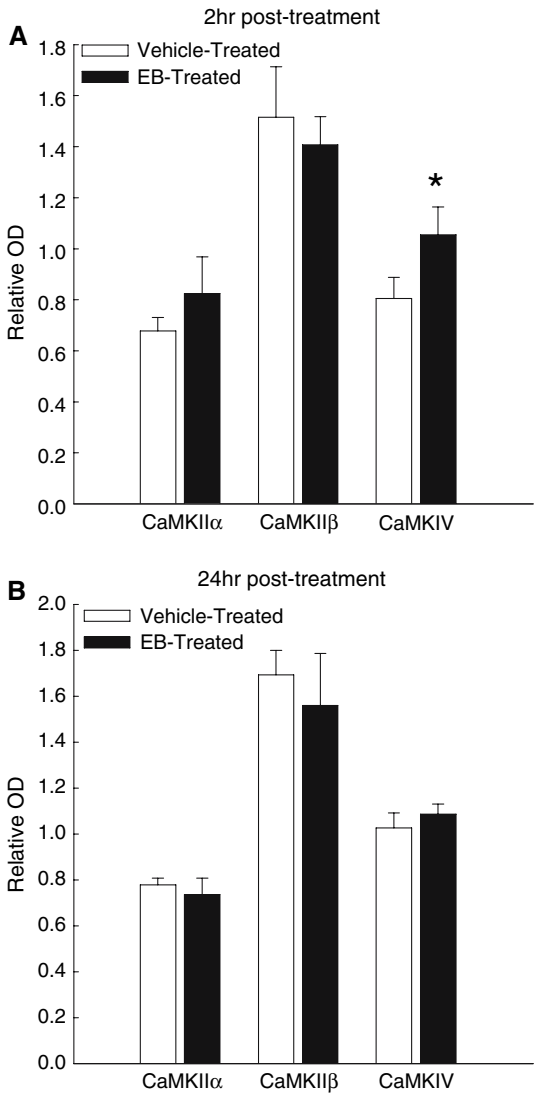

Fig. 3 Mean (±SEM) ROD of CaMKIIα, CaMKII*β*, and CaMKIV protein levels in the amygdala from EB-treated and vehicle-treated mice killed 2 h (**a**), and 24 h (**b**) after treatment. 15 μg of total protein from the amygdala was analyzed by SDS–PAGE electrophoresis and blotted to nitrocellulose membrane. Mean protein levels (ROD) are relative to *β*-actin. EB treatment did not alter CaMKIIα or CaMKII*β* protein levels within the amygdala 2- or 24 h after injection, but significantly increased CaMKIV protein levels 2 h after injection. Significant differences are denoted by an asterisk (*)

regulate neuronal excitability throughout the brain by modulating inhibitory tone. Interestingly, the parvalbumin neurons make up approximately 40% of the neurons in the basolateral amygdala and probably function as feedback inhibitory neurons [40–42]. Moreover, both calbindin-D28k and calretinin, two other calcium-binding proteins, are colocalized with GABA neurons within the of the basolateral amygdala [43], suggesting that calcium-binding proteins play an important role in regulating the inhibitory neurons of the amygdala. Interestingly, Calbindin D9k was downregulated within the amygdala in the microarray analysis, suggesting that estrogens may, in part, regulate amygdala activity through modulation of calcium-binding proteins within interneurons of the amygdala. Given that our lab and others have demonstrated that estrogens modulate fear and anxiety, it is interesting to speculate on the functional significance of the present findings. CaMK

expression is thought to be restricted primarily to pyramidal neurons within the amygdala [44]. It is possible that the effects of estrogens on CaMK gene expression are indirectly modulated via estrogen's effects on inhibitory intereurons of the amygdala. A similar arrangement has been observed in the hippocampus where estrogen inhibits GABAergic interneurons and reduces inhibitory tone on CA1 neurons, promoting synapse formation [45], suggesting that this might be one mechanisms by which estrogens regulate neuronal activity of the amygdala. Although we concentrate on the amygdala's role in regulating fear and anxiety, it is important to note that this region is involved in numerous additional functions, including appetitive behaviors, social behaviors, drug addiction as well as endocrine regulation. Future studies that identify cell-type specific responses to estrogens will better elucidate the mechanisms through which this steroid hormone regulates amygdala function and its modulation of emotion.

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