Regulation of gene expression by 17β-estradiol in the arcuate nucleus of the mouse through ERE-dependent and ERE-independent mechanisms

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Abstract

17β-estradiol (E2) modulates gene expression in the hypothalamic arcuate nucleus (ARC) to control homeostatic functions. In the ARC, estrogen receptor (ER) α is highly expressed and is an important contributor to E2's actions, controlling gene expression through estrogen response element (ERE)-dependent and -independent mechanisms. The objective of this study was to determine if known E2-regulated genes are regulated through these mechanisms. The selected genes have been shown to regulate homeostasis and have been separated into three subsections: channels, receptors, and neuropeptides. To determine if ERE-dependent or ERE-independent mechanisms regulate gene expression, two transgenic mouse models, an ERα knock-out (ERKO) and an ERα knock-in/knock-out (KIKO), which lacks a functional ERE binding domain, were used in addition to their wild-type littermates. Females of all genotypes were ovariectomized and injected with oil or estradiol benzoate (E2B). Our results suggest that E2B regulates multiple genes through these mechanisms. Of note, *Cacna1g* and *Kcnmb1* channel expression was increased by E2B in WT females only, suggesting an ERE-dependent regulation. Furthermore, the NKB receptor, *Tac3r*, was suppressed by E2B in WT and KIKO females but not ERKO females, suggesting that ERα-dependent, ERE-independent signaling is necessary for *Tac3r* regulation. The adrenergic receptor *Adra1b* was suppressed by E2B in all genotypes indicating that ERα is not the primary receptor for E2B's actions. The neuropeptide *Tac2* was suppressed by E2B through ERE-dependent mechanisms. These results indicate that E2B activates both ERα-dependent and independent signaling in the ARC through ERE-dependent and ERE-independent mechanisms to control gene expression.

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Introduction

The steroid hormone 17β-estradiol (E2) is known to regulate gene expression throughout the brain. E2 primarily uses two classical nuclear receptors, estrogen receptor α (ERα, *Esr1*,) and ERβ (*Esr2*) to regulate gene expression [1]. In the classical ER-mediated mechanism, ligand binding to the receptor initiates receptor recognition of the estrogen response element (ERE) to regulate gene transcription. In addition to classical regulation of gene expression, E2 also functions through ERE-independent mechanisms. As reviewed in McDevitt et al. (2008), these mechanisms include ligand-independent ER signaling, rapid effects through a membrane-associated ER, and ERE-independent signaling through protein-protein interactions (AP-1, etc.) [2].

In the hypothalamus, E2 mediates numerous homeostatic functions including reproduction, energy homeostasis, core body temperature, fluid balance, motivational behaviors, and stress physiology by regulating central neural pathways. Many of these pathways originate in or pass through the arcuate nucleus (ARC) of the hypothalamus [1, 3-5]. In the ARC, ERα is highly expressed and is the primary receptor used by E2 to control many homeostatic functions [5, 6]. Few studies have examined the physiological effects of ERα-mediated, ERE-dependent, and ERE-independent signaling on hypothalamic (ARC) gene expression.

Recently, the development of an ERα knock-in/knock-out (KIKO) mouse model lacking a functional DNA-binding domain (no ERE binding) gives insight to nonclassical, ERα-mediated, ERE-independent signaling while retaining ERβ-mediated signaling and other extra-nuclear initiated pathways (GPER, Gq-mER) [7, 8]. While KIKO females are infertile due to an absence of the LH surge [9, 10], they exhibit similar body weights, feeding, activity, oxygen consumption, glucose homeostasis and hypothalamic leptin sensitivity compared to wild-type (WT) females, unlike their total ERKO counterparts [11]. However, recent data from our lab suggest that ERα-mediated ERE-independent signaling partially restores the post-ovariectomy (ovx) weight gain but is not sufficient to mediate E2's attenuation of this weight gain [12].

E2 control of homeostatic functions occurs, in part, through regulation of important genes in the ARC. However, only a few studies have examined which signaling mechanisms E2 utilizes to control the expression of these genes. The KIKO mouse model provides an appropriate tool to increase our understanding of how homeostatic genes are regulated by E2-induced ERα-mediated mechanisms in the ARC. In the ARC, E2 is known to regulate the expression of a variety of cation channels including calcium channels and potassium channels [13-16]. The expression and activity of these cation channels are involved in regulating ARC proopiomelanocortin (POMC) and neuropeptide Y (NPY) neurons and their neuronal excitability [1, 12, 14-20]. Furthermore, E2 is known to regulate the mRNA expression of signaling molecules such as calmodulin and phosphatidylinositol-4,5-biphosphate 3-kinase (PI3K) [16, 21-23] and neurotransmitter enzymes like tyrosine hydroxylase (TH) and glutamate decarboxylase [1, 24]. Many of these E2-regulated genes are involved in reproduction, energy homeostasis, and hormone receptor signaling [1, 3, 25-27].

E2 is also known to suppress or augment the expression of a variety of neuropeptides and receptors in the hypothalamus, depending upon the experimental paradigm. In rodents, E2 increases *Pomc* and suppresses *Npy* expression in the ARC [1] and increases the expression of growth hormone [28]. It is also well known that E2 differentially regulates steroid receptors in the hypothalamus such as ERα, ERβ, and progesterone receptor (*Pgr*) [1, 16, 29]. Other hypothalamic hormone and neurotransmitter receptors are regulated by E2 in the hypothalamus include growth hormone secretagogue receptor (*Ghsr*) [30] and serotonin receptor 2C receptor (5HT2c) [31]. The mechanism underlying the regulation of all of these genes is largely unknown. Our current study focused on the regulation of ARC gene expression by E2.

E2 also regulates ARC KNDy (Kisspeptin-Neurokinin B-Dynorphin) neuronal gene expression [32]. Kisspeptin is involved in mediating negative and positive feedback of E2 on the hypothalamic-pituitary-gonadal (HPG) axis and potentially has a role in energy homeostasis and core body temperature [33]. Previous studies indicated that the *Kiss1* gene is regulated by E2 through ERE-independent mechanisms in the mediobasal hypothalamus while dynorphin expression is ERE-dependent [34]. Thus, we used these genes as positive and negative controls for ERE-dependent and ERE-independent gene expression. Nothing is known about the mechanisms behind the regulation of Neurokinin B (NKB, *Tac2*) or the KNDy receptors (*Kiss1r, Tac3r*) by E2 in the ARC. Therefore, the objective of this study was to determine if E2-responsive, homeostatic genes involved in reproduction and energy homeostasis that have been identified to be E2-responsive in the hypothalamus are regulated by E2 in the ARC through ERE-dependent or ERE-independent mechanisms using ovx WT, KIKO, and ERKO females.

Experimental

Animal care

All animal procedures were in compliance with institutional guidelines based on National Institutes of Health standards and were performed with Institutional Animal Care and Use Committee approval at Rutgers University. Adult C57BL/6 mice were housed under constant photoperiod conditions (12/12 h light/dark cycle) and maintained at a controlled temperature (25°C). Animals were given low phytoestrogen chow diet (<75 isoflavone ppm, Lab Diet Advanced Protocol 5V75, St. Louis, MO, USA) and water *ad libitum*. Animals were weaned on postnatal day 21 (PD21). Genotype was determined by using PCR products of extracted DNA from ear clippings, using previously published protocols [9]. We used three genotypes of mice: WT, KIKO, and ERKO (provided by Dr. Ken Korach, NIEHS) [9]. Crossing heterozygous WT/KI males expressing the nonclassical ERα knock-in with WT/KO heterozygous females generated WT and KIKO females. Crossing heterozygous WT/KO males and females generated WT and ERKO females. WT females used in the experiments were littermates generated from both colonies.

Drugs

17β-estradiol benzoate (E2B) was purchased from Steraloids (Newport, RI, USA) and dissolved in ethanol (1mg/ml) prior to mixing in sesame oil (Sigma-Aldrich). Ketamine was

purchased from Henry Schein Animal Health (Melville, NY, USA) and used for sedation prior to sacrifice.

Ovariectomy

Adult females (7-22 weeks and > 14 g body weight) were bilaterally ovx under isoflurane anesthesia 7 days prior to sacrifice using sterile no-touch techniques according to the NIH "Guidelines for Survival Rodent Surgery." Animals were given a dose of analgesic [4 mg/kg carprofen (Rimadyl®)] one day following surgery for pain management. Animals typically lost 1-2 g of weight one day after surgery. Females were monitored daily and allowed to recover for 5 days prior to the first injection of E2B or oil. The active metabolite of E2B is 17β-estradiol. Females were injected in the morning at 1000 h on post-ovx days 5 and 6 and sacrificed on post-ovx day 7 in the morning at 1000 h.

Experimental design

Females of each genotype (WT, KIKO, ERKO) were ovx and separated into a control sesame oil-treated group (n=6-9 per genotype) and an E2B-treated group (n=6-9 per genotype). An E2B injection protocol was used that has been shown to alter gene expression in the hypothalamus [19]. Animals were injected subcutaneously at 1000 h on post-ovx day 5 with either 0.25 μg of E2B or sesame oil. On post-ovx day 6, a 1.5 μg dose of E2B or sesame oil was injected at 1000 h. We did not include intact females in our experimental design as neither ERKO nor KIKO females exhibit a normal estrous cycle, which makes it difficult to compare among intact WT, KIKO, and ERKO females [11]. Animals were sacrificed on post-ovx day 7 at 1000 h. Animals were sedated with ketamine (100 μl of 100 mg/ml stock, i.p.) and decapitated. Brains were removed and rinsed in ice-cold Sorensen's Phosphate Buffer (0.2 M sodium phosphate, dibasic; and 0.2 M sodium phosphate, monobasic) for 30-60 sec. The basal hypothalamus (BH) was cut using a brain slice matrix (Ted Pella, Inc., Redding, CA, USA) into 1-mm thick coronal rostral and caudal blocks corresponding to Plates 42 to 47 and Plates 48 to 53, respectively, from *The Mouse Brain in Stereotaxic Coordinates* [35]. The slices were transferred to a 50/50 Pyrogard water/RNA*later*® (Life Technologies, Grand Island, NE, USA) solution and fixed overnight at 4°C. The ARC tissue, found in two slices, was microdissected using a dissecting microscope, following our previous publications [1, 12, 14, 35]. The microdissected sections represent the entirety of the ARC tissue. Dissected tissue was stored at −80 °C until RNA extraction. Trunk blood was collected at sacrifice to measure plasma E2 levels. Uteri were removed and wet weight was recorded. Wet uterine weight (mg) is a sensitive measure of circulating E2 in WT mice.

Tissue extraction

RNA was extracted from ARC using Ambion RNAqueous® Micro Kits (Life Technologies, Inc., Carlsbad, CA, USA) according to the manufacturer's protocol, followed by DNase-I treatment to remove contamination by genomic DNA. RNA samples were run on a NanoDrop™ ND-2000 spectrophotometer (ThermoFisher, Inc., Waltham, MA, USA) to assess quantity, followed by an Agilent 2100 Bioanalyzer run using the RNA 6000 Nano Kit

(Agilent Technologies, Inc., Santa Clara, CA, USA) to assess quality. Samples with a RNA integrity number (RIN) > 8 were used for quantitative real-time PCR (qPCR).

Blood preparation

Whole trunk blood was centrifuged (1300 rpm at 4 °C for 30 min). The supernatant was subjected to an additional 15 minutes of centrifugation (4 °C at 1300 rpm), then the plasma supernatant was removed and stored at −20 °C until E2 analysis. E2 concentration of plasma was analyzed using Mouse Calbiotech ELISA at the Ligand Assay and Analysis Core of the University of Virginia's Center for Research in Reproduction [36]. The Calbiotech ELISA is specific to E2 detection.

Quantitative real-time PCR

cDNA was synthesized from 200 ng of total RNA using Superscript III reverse transcriptase (Life Technologies, Inc.), 4 μl 5x buffer, 25 mM $MgCl_2$, 10 mM dNTP (Clontech Laboratories, Inc., Mountain View, CA, USA), 100 ng random hexamer primers (Promega Corporation, Madison, WI, USA), 40 U/μl Rnasin (Promega), and 100 mM DTT in DEPC-treated water (Gene Mate, Bioexpress, Inc., Kaysville, UT, USA) in a total volume of 20 μl. Reverse transcription was conducted using the following protocol: 5 min at 25 °C, 60 min at 50 °C, 15 min at 70 °C. A 1:20 dilution of the cDNA was produced using nuclease-free water (Gene Mate) for a final cDNA concentration of 0.5 ng/μl and stored at −20 °C. BH tissue RNA, which contains the ARC, from a male mouse was used as a positive control. A negative tissue control (BH with no reverse transcriptase) was also used.

A Taqman® Low Density Array (TLDA) (Life Technologies, Inc.) was used to analyze ARC gene expression in WT females. This array consisted of Taqman® expression assays of genes known to be E2-regulated and/or involved in reproduction and energy homeostasis (see Table 1 for a listing of genes analyzed). Data presented graphically only include genes significantly regulated by E2B, with the exception of Gpr30. The TLDA was designed so each expression assay was run in triplicate, including the reference gene, *Actb,* and the internal control, *18S.* On each TLDA plate, one WT experimental female sample was run in duplicate and a calibrator sample (male BH) was run on the remaining wells. The same calibrator sample was run on each plate. Taqman® primers were ordered for all genes, and KIKO and ERKO samples were analyzed. Positive and negative tissue control samples and master mix (nuclease-free water) controls were added to each run. In KIKO and ERKO qPCR plates, an additional sample (termed "pool") was analyzed that included all the control oil samples from each respective genotype, to account for inter-plate variation (data from C_q values analyzed across plates). qPCR was performed on a StepOnePlus™ Real-Time PCR System (Life Technologies, Inc.) using Taqman® Gene Expression Master Mix. For qPCR, we used 4 μg of cDNA (equivalent to 2 ng of total RNA). The amplification protocol for all genes was as follows: a holding stage consisted of 2 min at 50 °C and 95 °C for 10 min, followed by a cycling stage of 95 °C for 15 sec (denaturing) and at 60 °C (annealing) for 1 min for 40 cycles.

In addition to the genes analyzed in the TLDA, we also analyzed the mRNA expression of four additional genes: *Adra1b, Cart, Ghsr* and *Chrm1*, which were found to be E2-regulated

in our preliminary investigations or from the literature [37, 38]. Primers for these genes were designed to span exon-exon junctions and were synthesized by Life Technologies, Inc., using Clone Manager 5 software (Sci Ed Software, Cary, NC, USA). See Table 2 for a listing of synthesized primers used for qPCR. For qPCR of these four genes, we used 4 μg of cDNA (equivalent to 2 ng of total RNA) amplified with either PowerSYBR® Green Master Mix (*Adra1b, Chrm1;* Life Technologies, Inc.) or SsoAdvanced™ SYBR Green (*Cart, Ghsr;* BioRad, Inc., Hercules, CA, USA) on CFX-Connect Real-time PCR Instrument (BioRad, Inc.). A standard curve was generated for each primer pair using serial dilutions of BH cDNA in triplicate. Efficiencies were calculated as a percent efficiency, listed in Table 2. Amplification protocol for Table 2 genes was as follows: initial denaturing 95 °C for 10 min (PowerSYBR®) or 3 min (SsoAdvanced™) followed by 40 cycles of amplification at 94 °C for 10 sec (denaturing), 60 °C for 45 sec (annealing), and completed with a dissociation step for melting point analysis with 60 cycles of 95 °C for 10 sec, 65 °C to 95 °C (in increments of 0.5 °C) for 5 sec and 95 °C for 5 sec. The reference genes used were β-*actin* (PowerSYBR®) and *Gapdh* (SsoAdvanced™). Positive, negative, and water blank controls were included in the qPCR plate design.

Analysis of qPCR was done using the comparative C_q method using a 1:20 diluted BH cDNA (equivalent to 2 ng of RNA) sample from a male as the calibrator [39, 40]. All values were normalized to oil controls and are expressed as relative mRNA expression. In all plates, we maintained a consistent threshold level, set at the lowest but steepest slope of the exponential curve. We calculated the linear quantity of target genes using the formula $2^{-\Delta C_q}$. Data are expressed as *n*-fold difference from the calibrator, normalized to oil controls. The *n*-fold difference was used for statistical analysis.

Statistical analyses

qPCR data from the TLDA in WT females were initially analyzed using Data Assist® software (Life Technologies, Inc.) to determine significant differences between oil- and E2B-treated samples using a *t*-test with a false discovery rate (Benjamini-Hochberg method) set to a $p < 0.05$. All further statistical analyses were performed using GraphPad® Prism software (GraphPad Software, Inc., La Jolla, CA, USA). Data were expressed as mean ± SEM and analyzed by a two-way ANOVA (genotype × treatment), followed by a *post-hoc* Bonferroni's multiple comparison test between oil- and E2B-treated groups within each genotype (WT, KIKO, ERKO). Uterine weights were analyzed using a two-way ANOVA (genotype × treatment) followed by Bonferroni's multiple comparison test between oil- and E2B-treated groups, within each genotype (WT, KIKO, ERKO). Plasma E2 levels of all three genotypes were pooled together for oil vs. E2B treatment analysis, as there was no genotype effect (data not shown), and analyzed using a Student's *t*-test. In all experiments, a $p < 0.05$ was considered to be significant. To determine relative gene expression among genotypes, we compared oil-treated WT, KIKO, and ERKO females. These data were analyzed using a one-way ANOVA followed by a *post-hoc* Bonferroni's multiple comparison test.

Results

Uterine weights and plasma E2 levels

Following sacrifice, we dissected out the uterus of each female to confirm the hypertrophic actions of E2, an ERα-mediated process [41]. Past studies in our lab have suggested that E2B (250 ng/dose) replacement every other day for 4 weeks significantly increased the uterine weight in WT females [12]. E2B significantly increased the uterine weight in WT females (Table 3; ANOVA: $F(2,24) = 34.75$, $p < 0.0001$), but did not increase uterine weight in KIKO and ERKO females. There was no significant difference in body weight at sacrifice for E2B-treated females in all genotypes. The age of sacrifice of females was as follows: WT: 12-23 weeks (average: 18.2 ± 1.0 weeks); KIKO: 7-23 weeks (average: 17.2 ± 1.6 weeks); ERKO: 7-23 weeks (average: 13.9 ± 1.8 weeks). There were no age-specific effects when we analyzed the $2^{-\Delta Cq}$ -values for all regulated genes across genotypes (data not shown).

We pooled plasma E2 data across genotypes (WT, KIKO, ERKO) because there was no genotype effect observed (data not shown). Plasma E2 concentrations were as follows: oil-treated: 5.2 ± 0.4 pg/ml; E2B-treated: 28.82 ± 7.0 pg/ml. There was a significant increase in plasma E2 levels 24 h post-injection between oil- and E2B-treated groups ($p < 0.001$).

E2B dose regulates ARC gene expression

E2B-treatment in ovx WT females significantly regulated the mRNA expression of 14 genes in the ARC (Table 4). These genes include those that function as cation channels, receptors for hormones and neurotransmitters, and neuropeptides. E2 replacement significantly suppressed the mRNA expression of Adra1b, Cart, Chrm1, Esr1, Kiss1, Pdyn, NKB (Tac2), and *Tac3r* and significantly augmented expression of *Cacna1g, Esr2, Ghsr, Kcnmb1, Kiss1r,* and *Pgr*. The remaining genes in Table 4 were not regulated by E2B in WT females.

After studying WT ARC mRNA expression, we then analyzed all the genes in ARC tissue from KIKO and ERKO females injected with either sesame oil or E2B using individual Taqman® assays or custom primers. In comparing the genotypes, we found that the mRNA expression of two cation channels, which are involved in neuronal excitability, were upregulated by E2B in WT females only: Cav3.1 (*Cacna1g*), a subunit of the T-type Ca^{2+} channels, (Figure 1A; ANOVA: $F(2,30) = 4.004$, $p < 0.05$); and *Kcnmb1*, the β1 regulatory subunit for Ca^{2+}-activated potassium channel (Figure 1B; ANOVA: $F(1,27) = 6.221$, $p < 0.05$). The mRNA expression of both genes was increased more than two-fold in WT females injected with E2B.

In addition, multiple receptors involved in reproduction and energy balance showed a change in gene expression with E2B treatment. The mRNA expression of the cholinergic muscarinic receptor 1 (*Chrm1*) was suppressed threefold (Figure 2A; ANOVA: $F(2,30) = 25.64$; $p < 0.0001$), and ERα (*Esr1*) by ~35% (Figure 2B; ANOVA: $F(2,29) = 4.143$; $p < 0.05$) in WT females. E2B increased expression of growth hormone secretagogue receptor (*Ghsr*) (Figure 2C; ANOVA: $F(2, 30) = 28.45$; $p < 0.0001$) and progesterone receptor (*Pgr*) (Figure 2D; ANOVA: $F(2,29) = 13.43$; $p < 0.0001$) in WT females. Expression of *Tac3r,* the NKB receptor, was suppressed twofold in both WT and KIKO females by E2B (Figure 2E;

ANOVA: $F_{(1,29)} = 40.58$, $p < 0.0001$). Expression of adrenergic receptor, *Adra1b,* was suppressed by E2B across all genotypes (Figure 2F; ANOVA: $F_{(1,34)} = 17.64$, $p < 0.001$). Expression of the kisspeptin receptor, *Kiss1r,* was increased by E2B across all genotypes (Figure 2G; ANOVA: $F_{(1,29)} = 35.49$, $p < 0.0001$). Finally, ERβ (*Esr2*) expression was augmented by E2B in both WT and KIKO females (Figure 2H; ANOVA: $F_{(2,28)} = 4.452$, $p < 0.05$). GPER/GPR30 expression was not regulated by E2 in the ARC of any genotype (Figure 2I).

E2B also regulated several important neuropeptide genes in the ARC. In WT females only, prodynorphin (*Pdyn*) and neurokinin B (*Tac2*) expression were suppressed by E2B twofold (Figure 3A: ANOVA: $F_{(1, 29)} = 9.322$, $p < 0.01$) and fivefold (Figure 3B; ANOVA: $F_{(2, 29)} = 5.183$, $p < 0.05$), respectively. Kisspeptin (*Kiss1*) gene expression was suppressed fivefold in WT females and twofold in KIKO females (Figure 3C; ANOVA: $F_{(1,28)} = 27.63$, $p < 0.0001$). Interestingly, *Cart* expression was suppressed by E2B in WT and KIKO females, but was increased by E2B in ERKO females (Figure 3D; ANOVA: $F_{(2,42)} = 7.719$, $p < 0.01$). Surprisingly, we did not find a significant change in other E2-regulated arcuate genes including POMC and TH, which may be due to differences in treatment paradigms or rodent models [1, 42, 43].

ARC genes are differentially expressed in KIKO and ERKO ovx oil-treated females

Differences in gene expression across these genotypes may provide insight into signaling and feedback mechanisms involved in ERE-dependent, ERE-independent and ERα-independent signaling in the ARC. To determine if mRNA expression of the selected genes was different between the genotypes, we calculated the relative mRNA expression for each gene by normalizing the KIKO and ERKO oil groups to the WT oil group. Data were analyzed using a one-way ANOVA followed by *post-hoc* Bonferroni's multiple comparison tests between WT vs. KIKO, WT vs. ERKO, and KIKO vs. ERKO females. See Table 5 for results.

The mRNA levels of multiple genes were lower in KIKO and ERKO oil-treated females as compared to WT females. Interestingly, *Cart* expression was lower in KIKO females but not ERKO females compared to WT females. *Cart* expression in KIKO females was lower than in ERKO females (ANOVA: $F_{(2, 14)} = 19.43$, $p < 0.0001$). For eight genes, mRNA expression in KIKO and ERKO females was lower compared to WT females, with no difference observed between KIKO and ERKO females. These genes include: *Abcc8* (the regulatory subunit for K_{ATP} channels; ANOVA: $F_{(2,14)} = 15.63$, $p < 0.001$)*, Chrm1* (ANOVA: $F_{(2,14)} = 5.657$, $p < 0.05$), *Gad2* (glutamate decarboxylase 2; ANOVA: $F_{(2,14)} = 181.8$, $p < 0.0001$)*, Htr2c* (the 5HT2c serotonin receptor, ANOVA: $F_{(2,14)} = 30.72$, $p < 0.0001$)*, Kiss1* (ANOVA: $F_{(2,14)} = 10.43$, $p < 0.01$)*, Pomc* (ANOVA: $F_{(2,14)} = 12.48$, $p < 0.001$)*, Tac2* (ANOVA: $F_{(2,14)} = 26.99$, $p < 0.0001$), and *Th* (ANOVA: $F_{(2,14)} = 47.44$, $p < 0.0001$)*. For another two genes, mRNA expression was lower in KIKO and ERKO females compared to WT females, with mRNA expression also lower in ERKO females than in KIKO females. These genes were *Bcl2* (the anti-apoptotic gene B cell leukemia/ lymphoma 2; ANOVA: $F_{(2, 14)} = 124.8$, $p < 0.0001$), and *Sirt1* (Sirtuin 1 or NAD-dependent deactylase; ANOVA: $F_{(2, 14)} = 16.58$, $p < 0.001$).

The mRNA expression of three other genes was lower in ERKO females, but not KIKO females, compared to WT females. These genes include *Gpr30* (ANOVA: $F_{(2, 14)} = 10.05$, $p < 0.01$), *Kcnmb4* (Ca^{2+}-activated potassium channel subunit β4; ANOVA: $(2, 14) = 10.04$, p 0.01), and *Mtor* (mammalian target of rapamycin; ANOVA: $F_{(2, 14)} = 10.19$, $p < 0.01$). Additionally, six genes were expressed at lower levels in ERKO females compared to both WT and KIKO females. These genes were *Cacna1h* (a subunit of the T-type Ca^{2+} channels; ANOVA: $F_{(2, 14)} = 10.92$, $p < 0.001$), *Esr1* (ANOVA: $F_{(2,14)} = 22.96$, $p < 0.0001$), *Gad1* (glutamate decarboxylase 1; ANOVA: $F_{(2,14)} = 15.48$, $p < 0.001$), *Kcnj11* (Kir6.2, the channel subunit for K_{ATP} channels; ANOVA: $F_{(2,14)} = 20.18$, $p < 0.0001$), *Ncoa* (a coactivator of transcription; ANOVA: $F_{(2,14)} = 15.17$; $p < 0.001$), and *Pik3r3* (PI3K p55γ subunit; ANOVA: $F_{(2,14)} = 6.116$, $p < 0.05$). Interestingly, expression of calmodulin (*Calm1*) was lower in ERKO females compared to KIKO females, but not WT females (ANOVA: $F_{(2,14)} = 5.163$, $p < 0.05$).

We observed other gene-expression differences between genotypes. *Adra1b* expression was higher in KIKO females compared to WT females (ANOVA: $F_{(2,14)} = 10.82$, $p < 0.01$). Additionally, expression of *Cacna1g* (ANOVA: $F_{(2,14)} = 14.64$, $p < 0.001$) and *Pgr* (ANOVA: $F_{(2,14)} = 14.04$, $p < 0.001$) was higher in both KIKO and ERKO females compared to WT females. Expression of *Kiss1r* (ANOVA: $F_{(2,12)} = 16.29$, $p < 0.001$) and *Kcnmb1* (ANOVA: $F_{(2,14)} = 18.41$, $p < 0.001$) was higher in ERKO females compared to both WT and KIKO females. Interestingly, expression of *Pdyn* was higher in KIKO females compared to WT females and lower in ERKO compared to KIKO females (ANOVA: $F_{(2,14)} = 16.41$, $p < 0.001$). Finally, there was no change observed in *Esr2, Ghsr, Npy, Tac3r,* and *Kcnq5* (a subunit of the potassium channel that produces the M-current) in the ARC across oil-treated females in WT, KIKO, and ERKO genotypes.

Discussion

Previous studies have demonstrated that E2 regulates ARC gene expression to control homeostatic functions through ERα-mediated mechanisms. In the present study, to distinguish between ERE-dependent and ERE-independent transcriptional mechanisms, we compared gene expression in WT, ERα KO and an ERα KIKO mouse models. Previously, this KIKO model, which lacks ERα-mediated ERE-dependent signaling, was has been used to delineate such ERα-mediated signaling in the uterus [44] and the HPG axis [34, 45]. Studies on osteoblasts suggested that E2 regulation of gene transcription occurs through both ERE-dependent and ERE-independent mechanisms (including non-genomic mechanisms) [46]. The classical ERα signaling pathway is genomic ERE-dependent gene transcription in which E2 binds to ERα in the nucleus and then ERα binds to ERE to regulate expression of multiple genes. In addition to the classical ERα regulation of gene expression, there are ERE-independent mechanisms that include ERα/β-mediated, non-genomic second messenger pathways and ERα/β-independent signaling through membrane estrogen receptors [7, 8]. ERE-independent signaling includes PI3K and mitogen-activated protein kinase second messenger signaling cascades from membrane-associated ERα/β, protein-protein interactions with other transcription factors and ligand-independent mechanisms [2].

Recent evidence suggests that E2 signals through multiple membrane ERs including GPER (GPR30) [1, 3]. However, there are few studies that determine how E2 signals through ERE-dependent and ERE-independent mechanisms to regulate gene expression in the hypothalamus. This present study compared gene expression in WT, KIKO, and ERKO mouse models to characterize mechanisms of E2 regulation of ARC gene expression. We determined that regulation of ARC gene expression by E2B, with E2 being the active hormone, occurs through both ERE-dependent and ERE-independent mechanisms. The genes regulated by ERα-mediated, ERE-dependent and ERE-independent mechanisms in this study include cation channels, receptors, and neuropeptides associated with reproduction, energy balance, stress, and other homeostatic functions. However, it is necessary to note that our ERE-independent KIKO mouse model is nonselective to other hormone response element (HRE) motifs and may bind to other HREs to regulate transcription [47]. The development of an "EAAE" ERα mouse that lacks ERE- and HRE-dependent signaling would be useful in future studies to distinguish between those two types of signaling [47].

In the current study, E2 (or treatment with E2B) increased channel expression of *Cacna1g* and *Kcnmb1* in WT females only, a finding supported by previous studies, but not in KIKO or ERKO [16, 17, 19]. Voltage-dependent T-type calcium channel subunit alpha-1G (*Cacna1g*, also known as *Cav3.1*) is one subunit of low voltage-activated (T-type) calcium channels that is important in burst firing and neurotransmitter release. The elevated expression of *Cacna1g* expression in KIKO and ERKO females suggests that ERα-mediated, ERE-mediated signaling independent of ligand differentially regulates Cacna1g expression. Past studies have suggested that E2 regulates expression of the *Cav3* subunits [13, 15]. For example, in GnRH neurons of the preoptic area (POA), a high E2B dose increased *Cacna1g* expression transiently [13]. Increased expression of *Cacna1g* in the ARC led to increased neuronal excitability and burst firing to regulate hypothalamic neurons involved in reproduction and energy homeostasis [13, 15].

E2 has been found to regulate mRNA expression of a range of potassium channels in the guinea pig [16]. In the current study, we found that expression of *Kcnmb1*, a calcium-activated potassium channel (a MaxiK channel), was increased by E2B treatment in WT females only. Collectively, these studies emphasize the role of E2 regulation of channel expression in the ARC, which may translate to regulation of electrophysiological properties of ARC neurons [48]. Future studies in our lab will investigate the neurophysiological effects of the E2-induced gene expression. Furthermore, the results of our study indicate that in the mouse ARC, *Kcnmb1* MaxiK channel expression is regulated through ERE-dependent mechanisms. Elevated expression of *Kcnmb1* in oil-treated ERKO females also indicates that the loss of ERα positively affects ARC *Kcnmb1* gene expression.

In addition to the above findings related to channel expression regulation, the results of this study indicate that, through multiple ER-mediated mechanisms, E2B regulates several hormone and neurotransmitter receptors in the ARC. These receptors are involved in a number of functions including reproduction, neuronal excitability, and energy balance. Collectively, our data suggest that E2B regulates the expression of several receptors involved in feeding, stress, and neurotransmission through multiple receptor-mediated

mechanisms. *Chrm1* is a muscarinic acetylcholine receptor involved in a number of functions including memory consolidation, neuronal excitability and signal transduction [49]. Only a few studies to date have examined *Chrm1* expression in the ARC. In the present study, E2B suppressed *Chrm1* expression in the ARC through ERE-dependent signaling. Past studies in the rat hippocampus also suggest that *Chrm1* is decreased in response to immediate E2 replacement following ovx [50]. The current study used a different E2 replacement paradigm, yet it would be informative to examine *Chrm1* expression at different time points after E2B administration in the ARC to determine the time-dependent regulation of *Chrm1* expression. We also found suppressed *Chrm1* expression in KIKO and ERKO females compared to WT. This difference may result in a decrease in muscarinic signaling in the ARC of KIKO and ERKO females, which can be examined in future experiments using electrophysiology to assess muscarinic activity in ARC neurons. Furthermore, the role of muscarinic receptors in the control of homeostatic functions in the ARC has not been previously investigated and the effects of the suppression by E2 in these functions are unknown.

Expression of another neurotransmitter receptor, *Adra1b,* was suppressed by E2B treatment in WT, KIKO and ERKO females. Alpha-1B adrenergic receptor (*Adra1b)* is a receptor for catecholamines (norepinephrine/epinephrine) involved in arousal, feeding behaviors, cell growth, and proliferation. Previous studies in rats report that E2 increases *Adra1b* expression in the hypothalamus and POA [51-53]. E2 was administered via subcutaneous injection 24- and 48-hours prior to sacrifice, similar to the present study. The difference between those studies and the current study indicate the differential effects of E2 on adrenergic signaling between rodent species and between discrete hypothalamic nuclei. Furthermore, previous reports suggest that E2 inhibits catecholamine secretion *in vitro* [54, 55]. It should be noted that *Adra1b* suppression occurs in ERKO animals, suggesting that this is an ERα-independent mechanism. A decrease of *Adra1b* expression in the ARC would suppress, in part, the actions of noradrenergic/adrenergic signals from the hindbrain that control hypothalamic functions including arousal, feeding behavior, and energy expenditure [56, 57]. Genotype-specific analysis indicates that *Adra1b* expression and activity is different between KIKO and WT females, which may be a mechanism behind the differences in feeding behavior between the genotypes [12]. Future studies should examine if the difference in expression correlates with changes in noradrenergic signaling in ARC neurons, specifically NPY neurons.

The growth hormone secretagogue receptor, GHSR, is a GPCR involved in ghrelin signaling. E2B treatment has been shown to increase *Ghsr* expression in the ARC in mice [30]. We determined that the increase in ARC *Ghsr* expression by E2, which is regulated by ERE-dependent mechanisms, as E2B-treatment did not affect *Ghsr* expression in KIKO or ERKO females. Ghrelin signaling in the hypothalamus illuminates the relationship of E2 on feeding behavior control by ARC neurons. *Ghsr* activation by ghrelin excites NPY neurons [58] and initiates a signaling cascade that increases transcription of NPY and AgRP [59]. Activation of NPY neurons is associated with an increase in food intake, which is opposed by the actions of E2 [60]. However, *Ghsr* is expressed throughout the heterogeneous population of ARC neurons including POMC, TH, and KNDy neurons [30, 61, 62], and the

increase in ARC *Ghsr* expression by E2 is most likely involved in other homeostatic functions of these neurons. Cell-type specific analysis will be necessary to address this contradictory information of E2's regulation of GHSR signaling in the ARC.

Our results also indicate that steroid receptors associated with reproduction are regulated by E2 through both ERE-dependent and ERE-independent mechanisms. The classical role of E2 in the control of reproduction is through feedback mechanisms of the HPG axis and regulation of steroid receptor expression. The results of this study agree with past studies on E2 regulation of the estrogen receptor, ERα (*Esr1*). In the hypothalamus, E2 has been shown to decrease expression of *Esr1* [63]. This is supported by our study, which suggests that the E2-mediated *Esr1* suppression occurs through ERE-dependent mechanisms. There are no previous studies that identify the mechanism for this E2-mediated *Esr1* decrease. Furthermore, E2B treatment augmented the expression of ERβ (*Esr2*) in WT and KIKO females. These data indicate that, unlike with ERα, ERβ is regulated in the ARC by ERE-independent, ERα-mediated signaling. GPER/GPR30, a membrane estrogen receptor, was not regulated by E2 in any genotype. ERα (*Esr1*) expression was lower in ERKO compared to WT and KIKO females. GPER (*Gpr30*) expression was lower in ERKO compared to WT females. Lastly, there was no difference in expression of ERβ (*Esr2*) between all genotypes.

In addition, E2B treatment increased expression of *Pgr* in WT females only. These results are consistent with multiple studies that suggest E2B increases *Pgr* in the hypothalamus, priming the brain for progesterone's reproductive and behavioral actions [64, 65], but also suggest that *Pgr* is regulated through ERE-dependent, ERα-mediated signaling pathway. *Pgr* expression is two- to threefold higher in both KIKO and ERKO females compared to WT females. Interestingly, the relative expression of *Pgr* in oil-treated KIKO and ERKO females is equal to the relative levels of *Pgr* in E2B-treated, WT females. Higher expression of *Pgr* in the ARC may play a role in the lack of normal estrous cycles in KIKO and ERKO females.

In the present study, E2B treatment regulated multiple neuropeptides that are coexpressed in KNDy neurons of the ARC and their receptors. Kisspeptin-expressing neurons are expressed in two main regions in the rodent hypothalamus [32]. The first region is the anteroventral periventricular (AVPV) nucleus, which is referred to as the surge center for its role in the LH surge in female rodents [32]. The second region is the ARC, which contains kisspeptin neurons that coexpress Kisspeptin (*Kiss1*), Neurokinin B (*Tac2*) and Dynorphin (Dyn, *Pdyn*) [66]. These KNDy neurons are known for their contribution to negative feedback of E2 on the HPG axis and are hypothesized to be the pulse generator for the secretion of GnRH into the median eminence [67, 68]. Additional studies suggest that kisspeptin neurons directly contact GnRH neurons in the median eminence to control GnRH excitability and pulsatility [68]. Since then, KNDy neurons have also been shown to integrate feeding signals through input from peptides such as ghrelin, leptin, and insulin [69, 70]. Our results corroborate previous data demonstrating that *Kiss1* regulation by E2 is nonclassically mediated [34]. *Kiss1* expression also was lower in both KIKO and ERKO females than WT females. Because ARC *Kiss1* is involved in negative feedback, perhaps the lower expression indicates that negative feedback is disrupted in these genotypes, in part, due to lower expression of *Kiss1* [67, 68]..

Amongst the other two KNDy neuropeptides, Neurokinin B (*Tac2)* expression is suppressed by E2 in WT females only. Our data suggest that *Tac2* expression is primarily controlled by ERE-dependent signaling much like *Pdyn* [29, 41]. As with *Kiss1*, *Tac2* expression is lower in both KIKO and ERKO females. The suppressed expression of *Tac2* in these genotypes also supports the hypothesis that lower expression of both *Kiss1* and *Tac2* play a role in the disruption of negative feedback in these genotypes. Because neurokinin B (NKB, *Tac2*) is involved in GnRH pulse generation, the lower expression in KIKO and ERKO contributes to the dysregulation of the GnRH actions on the gonadotropes in these genotypes [67,68]. Finally, *Pdyn* expression, the other KNDy neuropeptide, is suppressed by E2B through ERE-dependent transcription. These data support previous studies and was used in this study as a "negative" control for ERE-independent signaling [34]. Interestingly, *Pdyn* expression was higher in KIKO females compared to WT females unlike *Kiss1* and *Tac2* expression. Elevated *Pdyn* may further disrupt the pulse generator in this genotype as this neuropeptide is considered a negative regulator of the pulse generator [67,68]. Because *Kiss1* is the only KNDy gene that is regulated by ERa ERE-independent mechanisms, the KNDy pulse generator (NKB, Dyn) for GnRH secretion is primarily controlled by ERE-dependent mechanisms.

KNDy-associated receptors are also regulated by E2. *Tac3r,* the NKB receptor, is suppressed in WT and KIKO females by E2B treatment, suggesting that, like *Kiss1, Tac3r* suppression is through an ERα-mediated, ERE-independent pathway [71]. These results suggest that both ERE-dependent and ERE-independent mechanisms are involved in the control of KNDy neuropeptides and their receptors and are necessary to maintain a functional HPG axis. In fact, current evidence suggests that mutations in *Tac3r* lead to problems in reproductive development including hypogonadotrophic hypogonadism, similar to mutations in *Kiss1r* [72, 73]. The decrease in *Tac3r* and *Tac2* associated with E2B treatment may both be involved in KNDy-mediated control of negative feedback and the GnRH pulse generator.

Kiss1r*,* the receptor for Kiss1*,* is increased by E2B treatment in all three genotypes. This upregulation suggests that E2 regulation of *Kiss1r* expression is ERα-independent and potentially mediated by ERβ, GPER, or perhaps the putative STX-responsive, Gq-coupled mER (Gq-mER) [1, 16, 74]. Alternatively, the increase in *Kiss1r* expression may be due, in part, to a decrease in the desensitization of the receptor, as Kiss1 peptide and gene expression is highly reduced by E2 in the ARC [25, 32, 75]. Future studies should examine E2 regulation of Kiss1r peptide expression selectively in the median eminence, where KNDy neurons contact GnRH neurons expressing the kisspeptin receptor [19, 66]. *Kiss1r* expression was higher in ERKO females compared to their WT and KIKO females, which correlated with a decrease in the ligand expression in the ERKO. However, the effect of E2 across the genotypes was similar, suggesting that the E2-mediated increase is robust and not dependent on relative baseline expression levels.

Lastly, *Cart* gene expression, which produces a neuropeptide involved in reward, stress, and feeding behavior [76, 77], is differentially regulated by E2 in the three genotypes. CART activity has been shown to suppress feeding much like E2 in rodents and primates [76, 77]. However, in this experiment, E2B treatment suppressed *Cart* in WT and KIKO females.

These results are supported by previous studies that have reported the suppression of *Cart* by E2 in the rodent ARC [38] and suggest that the suppression of the *Cart* gene in the ARC is a compensatory homeostatic response for the increase in CART protein activity in the paraventricular nucleus to suppress feeding [78]. Interestingly, E2B augmented *Cart* expression in ERKO females. The mechanism behind this effect of E2B is unknown, but certainly involves the other ER in the ARC. To date, there have been no studies that examine which ER-mediated mechanism regulates *Cart* expression or their contribution to the physiological effects of CART in the hypothalamus.

There are a number of additional receptors that can be involved in ERα-independent, E2 signaling and may be involved in the control of *Kiss1r*, *Adra1b*, and *Cart*. In addition to ERα, the classical receptor ERβ can also regulate E2-responsive genes through ERE-dependent and ERE-independent signaling. Although ERα is more highly expressed in the ARC compared to ERβ, studies suggest that ERβ signaling is still present [27] and may function as a compensatory mechanism for gene regulation by E2 in the ERKO since *Esr2* is expressed in ARC during development [79]. In addition, non-nuclear ER-mediated E2 signaling can also regulate gene expression through G-protein coupled membrane estrogen receptors (GPER) and the putative Gq-mER [80]. Evidence suggests that GPER is activated by E2 and is important for rapid cell signaling and homeostatic functions, although is it not regulated by E2 in the ARC [81, 82]. Gq-mER activates PLC-PKC signal transduction pathway and functions in energy homeostasis, bone remodeling, and core body temperature including activation of hypothalamic neurons [7] as well as control of ARC gene expression [1].

We found that there were a number of genes that were not regulated by E2B but did show differences in expression amongst genotypes. These genes include Abcc8, Bcl2, Cacna1h, Calm1, Gad1, Gad2, Gpr30, Htr2c, Kcnj11, Kcnmb4, Mtor, Ncoa1, Pik3r3, Pomc, Sirt1, and *Th.* The range of expression differences amongst the genotypes differs for each gene. While there is no easily discernable pattern of expression differences, these changes may result in deleterious effects of ERα loss (knockout) on reproductive and energy homeostasis. Furthermore, it is important to note that the present study only examined differences in gene expression among oil-treated genotypes, which may not produce functional differences in ARC neurons or in the protein of these enzymes, signaling molecules, receptors, and cation channels. Future experiments will be needed to confirm these potential effects and if these differences are due to a developmental role of ERα signaling, both ERE-dependent and ERE-independent, in ARC gene expression. Nonetheless, comparison of gene expression across genotypes is an important tool for enhancing our understanding of ERα's role in ARC gene expression and homeostatic functions.

While past studies have examined regulation of gene expression by E2 in the ARC, few studies to date have used the ERα KIKO transgenic mouse model as a tool to identify molecular mechanisms of E2 regulation. The results of our study indicate that genes involved in hypothalamic functions such as reproduction, energy homeostasis, and neuronal excitability are regulated by E2 through multiple ERα-mediated and ERα-independent pathways as has been previously suggested [1]. It is important to note that gene expression in our study is a measurement of the steady-state mRNA expression. Unfortunately, due to

the small size of the ARC nucleus in mice, it is not possible to conduct immunoblotting of protein expression within a single animal. Because mRNA and protein levels seldom correlate when comparing gene expression and immunohistochemistry [25], future studies will examine the expression and activity of many of these genes, especially the cation channels and GPCRs, using electrophysiology. These experiments would further characterize the role of ERα-mediated, ERE-dependent and -independent signaling on ARC gene expression and neuronal functions. Determining these signaling pathways is key to understanding the physiological effects of estrogens during the reproductive cycle and in hormone replacement therapies.

Acknowledgements

Abbreviations

Abcc8	ATP-binding cassette, subfamily C
Actb	beta actin
Adra1b	alpha-1B adrenergic receptor
AgRP	agouti-related protein
ARC	arcuate nucleus
AVPV	anteroventral periventricular nucleus
Bcl2	B cell leukemia/lymphoma 2
BH	basal hypothalamus
Cacna1g	T-type, voltage-dependent, calcium channel alpha 1G subunit
Cart	cocaine- and amphetamine-regulated transcript
Chrm1	cholinergic muscarinic 1 receptor
E2	17β-estradiol
E2B	17β-estradiol benzoate
ER	estrogen receptor
ERE	estrogen response element
ERKO	ERα knock-out
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
Esr1	estrogen receptor alpha
Esr2	estrogen receptor beta

Gapdh	glyceraldehyde-3-phosphate dehydrogenase
Ghsr	growth hormone secretagogue receptor
GnRH	gonadotropin releasing hormone
GPCR	g-protein coupled receptor
GPER	G protein-coupled estrogen receptor 1
HPG	hypothalamic-pituitary-gonadal
HRE	hormone response element
Kcnmb1	calcium-activated potassium channel subunit β1
KIKO	knock-in/knock-out
Kiss1	kisspeptin
Kiss1r	kisspeptin receptor
KNDy	Kisspeptin-Neurokinin B-Dynorphin
Mtor	mammalian target of rapamycin
NPY	neuropeptide Y
ovx	ovariectomized
Pdyn	prodynorphin
Pgr	progesterone receptor
POMC	proopiomelanocortin
qPCR	quantitative real-time polymerase chain reaction
Tac2	tachykinin 2
Tac3r	tachykinin 3 receptor
TH	tyrosine hydroxylase
TLDA	Taqman Low Density Array
WT	wild-type

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Highlights

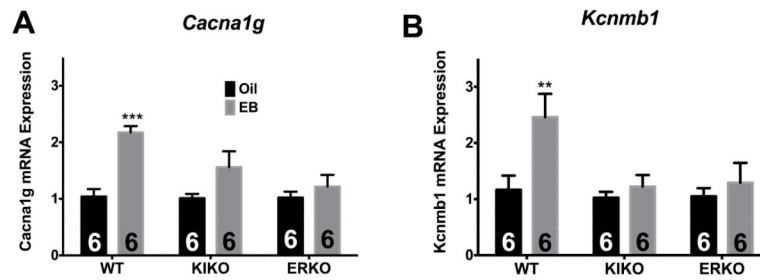- WT, ERKO, and KIKO females were used to study ERE-dependent and ERE-independent signaling.

- Gene regulation by E2B occurs through ERE-dependent and ERE-independent mechanisms.

- Gene expression varies greatly in oil-treated females between the genotypes.

- Channels, receptors, and neurotransmitters are regulated in the ARC.

Figure 1. E2B regulates channel gene expression in the ARC

(A) *Cacna1g* and (B) *Kcnmb1.* Results of qPCR analyses represent gene expression of oil- (black bars) and E2B- (gray bars) treated females in WT, KIKO, and ERKO genotypes. The number of animals in each treatment group is listed within each bar. Genes are expressed as relative *n*-fold changes, normalized to oil controls, within each genotype (WT, KIKO, ERKO). A two-way ANOVA (genotype × treatment) followed by *post-hoc* Bonferroni's multiple comparison test was used to determine significant differences between treatments, within genotype. **p < 0.01; ***p < 0.001.
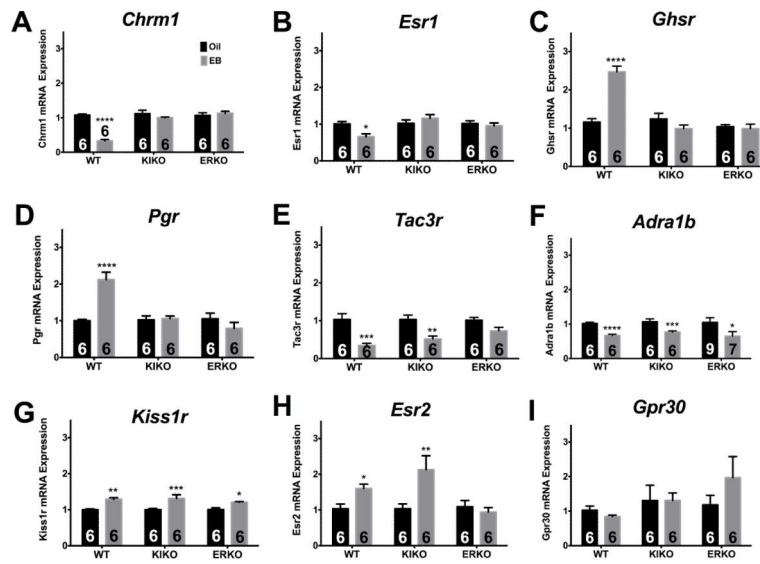
Figure 2. E2B regulates receptor gene expression in the ARC

(A) *Chrm1*, (B*) Esr1*, (C) *Ghsr*, (D) *Pgr,* (E) *Tac3r,* (F) *Adra1b,* (G) *Kiss1r*, (H) *Esr2,* and (I) *Gpr30.* Results of qPCR analyses represent gene expression of oil- (black bars) and E2B- (gray bars) treated females in WT, KIKO, and ERKO genotypes. The number of animals in each treatment group is listed within each bar. Genes are expressed as relative *n*-fold changes, normalized to oil controls, within each genotype (WT, KIKO, ERKO). A two-way ANOVA (genotype × treatment) followed by *post-hoc* Bonferroni's multiple comparison test was used to determine significant differences between treatments, within genotype. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.
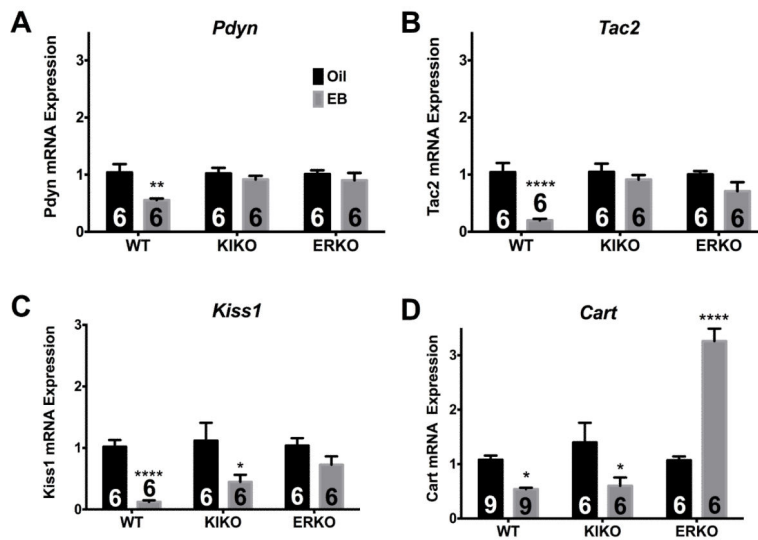
Figure 3. E2B regulates neuropeptide gene expression in the ARC
(A) *Pdyn,* (B) *Tac2,* (C) *Kiss1, and* (D) *Cart.* Results of qPCR analyses represent gene expression of oil- (black bars) and E2B- (gray bars) treated females in WT, KIKO, and ERKO genotypes. The number of animals in each treatment group is listed within each bar. Genes are expressed as relative *n*- fold changes, normalized to oil controls, within each genotype (WT, KIKO, ERKO). A two-way ANOVA (genotype × treatment) followed by *post-hoc* Bonferroni's multiple comparison test was used to determine significant differences between treatments, within genotype. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Table 1

List of genes analyzed in the Taqman® Low Density Array (TLDA)

Gene Name	Gene Abbreviation	Taqman Assay #
ATP-binding cassette, subfamily C	*Abcc8*	Mm00803450_m1
B cell leukemia/lymphoma 2	*Bcl2*	Mm00477631_m1
Cav3.1	*Cacna1g*	Mm00486572_m1
Cav3.2	*Cacna1h*	Mm00445382_m1
Calmodulin 1	*Calm1*	Mm01336281_g1
Estrogen receptor α	*Esr1*	Mm00433149_m1
Estrogen receptor β	*Esr2*	Mm00599821_m1
Glutamate decarboxylase 1	*Gad1*	Mm04207432_g1
Glutamate decarboxylase 2	*Gad2*	Mm00484623_m1
G protein-coupled receptor 30	*Gpr30*	Mm01194815_m1
Growth hormone	*Gh*	Mm00433590_g1
Serotonin receptor 2C	*Htr2c*	Mm00434127_m1
Calcium-activated potassium channel subunit β1	*Kcnmb1*	Mm00466621_m1
Kir6.2	*Kcnj11*	Mm00440050_s1
Calcium-activated potassium channel subunit β4	*Kcnmb4*	Mm00465684_m1
KCNQ5 (Kv7.5)	*Kcnq5*	Mm01226041_m1
Kisspeptin 1	*Kiss1*	Mm03058560_m1
Kisspeptin receptor	*Kiss1r*	Mm00475046_m1
Neuropeptide Y	*Npy*	Mm03048253_m1
Prodynorphin	*Pdyn*	Mm00457573_m1
Progesterone receptor	*Pgr*	Mm00435628_m1
PI3K p55γ	*Pik3r3*	Mm00725026_m1
Proopiomelanocortin	*Pomc*	Mm00435874_m1
Tachykinin 2	*Tac2*	Mm00436885_m1
Tachykinin 3 receptor	*Tac3r*	Mm00445346_m1
Tyrosine hydroxylase	*Th*	Mm00447557_m1
Nuclear receptor coactivator 1	*Ncoa*	Mm01318933_m1
Mammalian target of rapamycin	*Mtor*	Mm00444968_m1
Sirtuin 1	*Sirt1*	Mm00490758_m1
Beta actin	*Actb*	Mm01205647_g1
18s ribosomal RNA	*18S*	Hs99999901_s1

Genes that were analyzed using a TLDA and their respective Taqman® assay #s are listed. For KIKO and ERKO analysis, identical Taqman® assays were used.

Table 2

List of genes using designed primers

Gene Name	Product length	% Eff	Primer sequence	Base pair #	Accession #
Adra1b	84	100	F: CTTCATCGCTCTCCCACTTG	1174-1193	NM_007416
			R: TAGCCCAGCCAGAACACT	1240-1257	
β-actin	63	100	F: GCCCTGAGGCTCTTTTCCA	849-867	NM_007393.3
			R: TAGTTTCATGGATGCCACAGGA	911-990	
Cart	169	93	F: GCTCAAGAGTAAACGCATTCC	277-297	NM_013732
			R: GTCCCTTCACAAGCACTTCAA	425-445	
Chrm1	272	110	F: AGCAGCTCAGAGAGGTCACAGCCA	1331-1354	NM_001112697
			R: GGGCCTCTTGACTGTATTTGGGGA	1580-1603	
Gapdh	98	93	F: TGACGTGCCGCCTGGAGAAA	852-875	NM_008084
			R: AGTGTAGCCCAAGATGCCCTTCAG	106-125	
Ghsr	122	110	F: CAGGGACCAGAACCACAAAC	1003-1022	NM_177330
			R: AGCCAGGCTCGAAAGACT	1107-1124	

The genes listed are those that were not included in the TLDA. Sense primer is listed first with the antisense primer below. Primer pairs were ordered from Life Technologies and designed to span exon-exon junctions using Clone 5 software. *Adra1b,* alpha-1B adrenergic receptor; *β-actin,* beta actin; *Cart*, cocaine- and amphetamine- regulated transcript; *Chrm1*, cholinergic muscarinic 1 receptor; *Gapdh,* glyceraldehyde-3-phosphate dehydrogenase; *Ghsr*, growth hormone secretagogue receptor.

Table 3

Body and uterine weights

Genotype	Treatment	Body weight (g)	Uterine weight (mg)
WT	Oil	24.0 ± 0.6	27.5 ± 0.9
	E2B	23.6 ± 0.7	98.3 ± 2.1[****]
KIKO	Oil	22.1 ± 1.6	38.8 ± 5.3
	E2B	22.4 ± 0.9	43.0 ± 5.1
ERKO	Oil	19.5 ± 0.3	10.0 ± 1.3
	E2B	21.0 ± 0.9	13.2 ± 2.4

Uterine weights are expressed relative to body weight. Data were analyzed with a two-way ANOVA with Bonferroni's multiple comparison tests

[****] $p < 0.0001$

Table 4

TLDA Gene Expression Analysis

Gene Name	Functional Name (Protein)	Oil	E2B	p
Abcc8	ATP-binding cassette, subfamily C member 8	1.02 ± 0.10	0.97 ± 0.11	-
Adra1b	Alpha-1B adrenergic receptor	1.02 ± 0.04	0.67 ± 0.03	****
Bcl2	Apoptosis regulator Bcl-2	1.00 ± 0.03	0.99 ± 0.06	-
Cacna1g	Voltage-dependent T-type calcium channel subunit alpha-1G	1.01 ± 0.08	1.61 ± 0.17	***
Cacna1h	Voltage-dependent T-type calcium channel subunit alpha-1H	1.01 ± 0.05	1.03 ± 0.06	-
Calm1	Calmodulin	1.01 ± 0.07	1.13 ± 0.05	-
Cart	Cocaine- and amphetamine-regulated transcript protein	1.08 ± 0.08	0.54 ± 0.02	*
Chrm1	Muscarinic acetylcholine receptor M1	1.08 ± 0.03	0.33 ± 0.04	****
Esr1	Estrogen receptor α	1.01 ± 0.06	0.66 ± 0.08	*
Esr2	Estrogen receptor β	1.03 ± 0.13	1.60 ± 0.12	*
Gad1	Glutamate decarboxylase 1	1.01 ± 0.06	0.94 ± 0.07	-
Gad2	Glutamate decarboxylase 2	1.01 ± 0.05	1.03 ± 0.06	-
Gh	Growth Hormone (Somatotropin)	1.03 ± 0.11	1.01 ± 0.11	-
Ghsr	Growth hormone secretagogue receptor type 1	1.16 ± 0.09	2.47 ± 0.15	****
Gpr30	G-protein coupled estrogen receptor 1	1.03 ± 0.12	0.84 ± 0.04	-
Htr2c	5-hydroxytryptamine receptor 2C	1.01 ± 0.09	1.20 ± 0.14	-
Kcnj11	ATP-sensitive inward rectifier potassium channel 11	1.01 ± 0.07	0.92 ± 0.05	-
Kcnmb1	Calcium-activated potassium channel subunit beta-1	1.16 ± 0.25	2.45 ± 0.41	**
Kcnmb4	Calcium-activated potassium channel subunit beta-4	1.03 ± 0.11	1.30 ± 0.07	-
Kcnq5	Potassium voltage-gated channel subfamily KQT member 5	1.00 ± 0.04	1.12 ± 0.11	-
Kiss1	Kisspeptin	1.02 ± 0.11	0.12 ± 0.02	***
Kiss1r	Kisspeptin receptor	1.00 ± 0.02	1.29 ± 0.05	**
Mtor	Serine/threonine-protein kinase mammalian target of rapamycin	1.00 ± 0.04	1.08 ± 0.06	-
Ncoa	Nuclear receptor coactivator 1	1.00 ± 0.05	1.07 ± 0.04	-
Npy	Neuropeptide Y	1.13 ± 0.25	0.99 ± 0.19	-
Pdyn	Prodynorphin	1.04 ± 0.15	0.55 ± 0.03	**
Pgr	Progesterone receptor	1.00 ± 0.04	2.12 ± 0.20	****
Pik3r3	Phosphatidylinositol-4,5 bisphosphate 3-kinase regulatory subunit gamma	1.00 ± 0.03	1.12 ± 0.06	-
Pomc	Pro-opiomelanocortin	1.01 ± 0.08	0..94 ± 0.10	-
Sirt1	NAD-dependent protein deacetylase sirtuin-1	1.00 ± 0.04	1.06 ± 0.03	-
Tac2	Tachykinin 2 (Neurokinin B)	1.04 ± 0.16	0.19 ± 0.03	****
Tac3r	Tachykinin 3 receptor	1.04 ± 0.15	0.35 ± 0.06	****
Th	Tyrosine hydroxylase	1.04 ± 0.13	1.01 ± 0.05	-

Data were analyzed by a Students *t*-test for each gene

*
p < 0.05;

**
p < 0.01;

***
p < 0.001;

****
p < 0.0001

Table 5

Comparison of gene expression between genotypes in oil-treated females

Gene List	WT	KIKO	ERKO
Abcc8	1.02 ± 0.10	0.32 ± 0.14 [b]	0.21 ± 0.08 [c]
Adra1b	1.03 ± 0.12	3.97 ± 0.64 [b]	2.64 ± 0.29
Bcl2	1.00 ± 0.03	0.24 ± 0.07 [d]	0.02 ± 0.01 [d D]
Cacna1g	1.01 ± 0.08	1.97 ± 0.15 [b]	2.36 ± 0.24 [c]
Cacna1h	1.01 ± 0.05	0.82 ± 0.21	0.09 ± 0.02 [c B]
Calm1	1.01 ± 0.07	1.07 ± 0.35	0.18 ± 0.05 [A]
Cart	1.02 ± 0.10	0.19 ± 0.05 [d]	0.77 ± 0.12 [B]
Chrm1	1.10 ± 0.31	0.34 ± 0.07 [A]	0.34 ± 0.06 [A]
Esr1	1.01 ± 0.06	0.97 ± 0.09	0.44 ± 0.03 [c C]
Esr2	1.03 ± 0.13	1.80 ± 0.45	2.00 ± 0.32
Gad1	1.01 ± 0.06	0.66 ± 0.16	0.16 ± 0.06 [c A]
Gad2	1.01 ± 0.05	0.15 ± 0.03 [d]	0.09 ± 0.02 [A]
Ghsr	1.01 ± 0.07	1.58 ± 0.42	1.11 ± 0.14
Gpr30	1.03 ± 0.12	0.61 ± 0.21	0.12 ± 0.03 [b]
Htr2c	1.01 ± 0.09	0.26 ± 0.09 [d]	0.18 ± 0.07 [d]
Kcnj11	1.01 ± 0.07	0.83 ± 0.15	0.13 ± 0.06 [c C]
Kcnmb1	1.16 ± 0.25	6.87 ± 1.45	14.04 ± 1.91 [d A]
Kcnmb4	1.03 ± 0.11	0.50 ± 0.20	0.13 ± 0.06 [b]
Kcnq5	1.00 ± 0.04	0.85 ± 0.33	0.20 ± 0.06
Kiss1	0.92 ± 0.27	0.02 ± 0.01 [b]	0.17 ± 0.05 [A]
Kiss1r	1.48 ± 0.53	0.45 ± 0.11	6.84 ± 1.66 [b C]
Mtor	1.00 ± 0.04	0.54 ± 0.18	0.23 ± 0.06 [b]
Ncoa	1.00 ± 0.05	0.67 ± 0.16	0.18 ± 0.04 [c A]
Npy	1.13 ± 0.25	0.87 ± 0.49	0.45 ± 0.23
Pdyn	1.04 ± 0.15	2.39 ± 0.23 [c]	1.50 ± 0.10 [B]
Pgr	1.00 ± 0.04	3.42 ± 0.35 [c]	2.59 ± 0.38 [A]
Pik3r3	1.00 ± 0.03	1.01 ± 0.28	0.24 ± 0.09 [a A]
Pomc	1.01 ± 0.08	0.44 ± 0.17 [A]	0.17 ± 0.07 [c]
Sirt1	1.00 ± 0.04	0.59 ± 0.15 [A]	0.18 ± 0.02 [c A]
Tac2	1.08 ± 0.18	0.01 ± 0.01 [d]	0.13 ± 0.05 [c]
Tac3r	1.38 ± 0.83	0.05 ± 0.02	0.12 ± 0.03
Th	1.04 ± 0.13	0.23 ± 0.05 [d]	0.05 ± 0.01 [d]

Data were normalized to WT oil and analyzed by a one-way ANOVA followed by post-hoc Bonferroni's multiple comparison test, within gene. Lowercase letters denote changes observed between WT and KIKO or ERKO

Uppercase letters sign denotes changes observed between KIKO and ERKO females

[a]=p < 0.05;

[b]=p < 0.01;

[c]=p < 0.001;

[d]=p < 0.0001

[A]=p < 0.05;

[B]=p < 0.01;

[C]=p < 0.001;

[D]=p < 0.0001