Behavioral/Systems/Cognitive

Regulation of *Kiss1* and *Dynorphin* Gene Expression in the Murine Brain by Classical and Nonclassical Estrogen Receptor Pathways

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Kisspeptin is a product of the *Kiss1* gene and is expressed in the forebrain. Neurons that express *Kiss1* play a crucial role in the regulation of pituitary luteinizing hormone secretion and reproduction. These neurons are the direct targets for the action of estradiol-17β (E_2), which acts via the estrogen receptor α isoform (ERα) to regulate *Kiss1* expression. In the arcuate nucleus (Arc), where the *dynorphin* gene (*Dyn*) is expressed in *Kiss1* neurons, E_2 inhibits the expression of *Kiss1* mRNA. However, E_2 induces the expression of *Kiss1* in the anteroventral periventricular nucleus (AVPV). The mechanism for differential regulation of *Kiss1* in the Arc and AVPV by E_2 is unknown. ERα signals through multiple pathways, which can be categorized as either classical, involving the estrogen response element (ERE), or nonclassical, involving ERE-independent mechanisms. To elucidate the molecular basis for the action of E_2 on *Kiss1* and *Dyn* expression, we studied the effects of E_2 on *Kiss1* and *Dyn* mRNAs in the brains of mice bearing targeted alterations in the ERα signaling pathways. We found that stimulation of *Kiss1* expression by E_2 in the AVPV and inhibition of *Dyn* in the Arc required an ERE-dependent pathway, whereas the inhibition of *Kiss1* expression by E_2 in the Arc involved ERE-independent mechanisms. Thus, distinct ERα signaling pathways can differentially regulate the expression of identical genes across different brain regions, and E_2 can act within the same neuron through divergent ERα signaling pathways to regulate different neurotransmitter genes.

Introduction

Gonadotropin-releasing hormone (GnRH) neurons represent the final common pathway through which the brain regulates reproduction. The secretion of GnRH is controlled by the negative and positive feedback actions of 17β-estradiol (E_2), which in turn coordinate reproductive cycles. The primary effects of E_2 on GnRH secretion are mediated by estrogen receptor α (ERα) (Rissman et al., 1997; Shughrue et al., 1997, 2002; Couse and Korach, 1999); however, GnRH neurons do not express ERα (Herbison and Theodosis, 1992). Kisspeptin neurons in the hypothalamus are thought to relay information about estrogen status directly to GnRH neurons (Popa et al., 2008). Kisspeptin, which is coded by the *Kiss1* gene, is expressed in the rodent by neurons in the arcuate (Arc) and anteroventral periventricular (AVPV) nuclei that coexpress ERα (Smith et al., 2005). E_2 inhibits *Kiss1* expression in the Arc (Smith et al., 2005), a nodal point in negative feedback regulation of GnRH/luteinizing hormone (LH) secretion (Nishihara et al., 1986; Thind and Goldsmith, 1988; Goodman, 1996), and conversely, E_2 stimulates the expression of *Kiss1* in the AVPV (Smith et al., 2005), an essential area for generating the preovulatory GnRH surge through positive feedback (Wiegand et al., 1978; Terasawa et al., 1980; Popolow et al., 1981; Ronnekleiv and Kelly, 1988; Simerly et al., 1990; Gu and Simerly, 1997; Polston and Simerly, 2006). Although the induction and inhibition of *Kiss1* expression in the AVPV and Arc both require ERα (Smith et al., 2005), the molecular pathways through which ERα differentially regulates *Kiss1* expression in these regions remain a mystery.

ERα exerts its effects through multiple signaling pathways (O'Malley and Tsai, 1992; Glass, 1994; McKenna et al., 1999; Smith and O'Malley, 2004; McDevitt et al., 2008). One pathway involves the translocation of ERα into the nucleus, where it recruits cofactors to estrogen response element (ERE) regulatory sites to alter gene transcription—termed classical signaling (Glass, 1994; Tsai and O'Malley, 1994; McKenna et al., 1999). ERα signaling can also employ ERE-independent genomic pathways that entail interactions with other transcription factors—termed the nonclassical pathway (Gaub et al., 1990; Ray et al., 1994; Stein and Yang, 1995; Webb et al., 1995; Kushner et al., 2000; Jakacka et al., 2001, 2002). Recently, it has been shown that the positive feedback effects of E_2 require an ERE-dependent

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pathway, whereas negative feedback involves an ERE-independent mechanism (Glidewell-Kenney et al., 2007). We argued that if *Kiss1* in the AVPV mediates positive feedback, the induction of *Kiss1* by E_2 in the AVPV would require ERE-dependent signaling. Likewise, if *Kiss1* in the Arc mediates negative feedback, the inhibition of *Kiss1* and dynorphin [*Dyn*; which is coexpressed with *Kiss1* (Goodman et al., 2007) (V. M. Navarro, D. K Clifton, R. A. Steiner, our unpublished observations)] in the Arc would require an ERE-independent mechanism. To test this hypothesis and dissect the molecular pathways by which E_2 regulates *Kiss1* and *Dyn* gene expression in the brain, we studied mice with genetically engineered alterations in the ERα signaling pathways and analyzed the effects of E_2 on *Kiss1* and *Dyn* mRNAs (Glidewell-Kenney et al., 2007).

Materials and Methods

Animals. Animals were housed at Northwestern University and surgeries were conducted there according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animal use procedures were approved by the Northwestern University Animal Care and Use Committee. Nonclassical estrogen receptor knock-in (ER$\alpha^{AA/-}$) mice were created by crossing mutant mice that have an ERα allele that cannot bind ERE (E207A/G208A; AA) on a 129SvJ background with ER$\alpha^{-/-}$ mice. ER$\alpha^{AA/-}$ mutants used in these experiments are the result of the AA mutant allele crossed 7–11 generations onto the C57BL/6 background. ER$\alpha^{-/-}$ mice (obtained from Pierre Chambon, Collège de France, Illkirch, France) were on a C57BL/6 background. Mice were maintained on a 14 h light:10 h dark cycle with standard chow (7912; Harlan Teklad) and water available *ad libitum*.

Ovariectomy and estradiol replacement. Adult female mice (10–13 weeks of age) were ovariectomized on day 0. The animals were anesthetized by isoflurane inhalation. Immediately after ovariectomy (OVX), animals received midscapular subcutaneous SILASTIC capsules (1.5 cm in length that were plugged with silicone adhesive on each end to leave 1 cm to fill with treatment, inner diameter 1.47 mm; outer diameter 1.95 mm) that were either filled with only sesame oil (OVX + vehicle group; $n = 5$) or capsules containing 1 mg/ml E_2 in sesame oil (OVX + E_2 group; $n = 6$) (Miller et al., 1995; Smith et al., 2005; Dungan et al., 2007). Between 9:00 and 10:00 A.M. 7 d after OVX, animals were killed and brains and sera were obtained. Brain tissue was sent to the University of Washington for analysis. Serum measurements for E_2 and LH were conducted at Northwestern University.

Serum collection and assays. Blood was allowed to coagulate for 90 min at room temperature and centrifuged at 2000 × *g* for 15 min. Serum was transferred to a fresh tube and stored at −20°C. Serum levels of LH and E_2 were measured at Northwestern University (Evanston, IL). Reagents for the LH assay were from the National Institutes of Health, the antiserum used was anti-r-LH-S11, and the standard was rLH-RP3. The assay sensitivity was 0.2 ng/ml, and the intra-assay coefficient of variation was 4%. E_2 was measured with a double-antibody kit (Diagnostics Production). The assay sensitivity was 2.0 pg/ml, and the intra-assay coefficient of variation was 6%.

Tissue preparation. Mice were anesthetized with isoflurane and then killed by decapitation. Brains were removed and frozen on dry ice. Sections in the coronal plane (20 μm) were cut on a cryostat, thaw-mounted onto SuperFrost Plus slides (VWR Scientific), and stored at −80°C. Sections were collected from the diagonal band of Broca to the mammillary bodies.

Detection of Kiss1 *mRNA.* The *Kiss1* probe used for detection of *Kiss1* mRNA was previously described by Gottsch et al. (2004). The *Kiss1*-specific sequence of the probe spans bases 76–486 of the mouse cDNA sequence (GenBank accession no. AF472576). The procedure for *in situ* hybridization (ISH) is outlined below.

Detection of Dynorphin mRNA. Total RNA was extracted from mouse brain using an RNAqueous Kit (Ambion). RNA was reverse transcribed into cDNA with a RetroScript kit (Ambion) primed with oligodeoxythymidine (dT) for subsequent PCR. Primers were designed based on the published sequence of the *Pro-Dynorphin* mouse gene (GenBank accession number NM_018863) with forward primers starting at 132 bp and reverse primers starting at 540 bp for a product of ~409 bp. Primers were custom synthesized (OPERON). PCRs contained the following in a volume of 25 μl: 2 μl of reverse transcriptase reaction product; 0.2 μM of each primer; 12.5 μl of RediTaq polymerase (Sigma-Aldrich); and 8.5 μl of water. Reactions were performed in a PTC-100 thermal cycler (MJ Research) using the following protocol: cDNA was denatured for 5 min at 94°C, then 35 cycles were performed at 94°C for 1 min, 55°C for 1.5 min, and 72°C for 2 min, with a final 5 min extension at 72°C. After electrophoresis on a 2% agarose (w/v) gel, a single DNA fragment was obtained of approximately the expected size (409 bp) and gel purified with a QiaQuick gel extraction kit (QIAGEN). The PCR product was confirmed to be the mouse Dynorphin probe by sequencing. Clamp polymerase sequences for T7 or T3 polymerase were added for the final primer product sequence of *Dyn* forward primer: CAGAGATGCAATTAACCCTCAACTAAAGGGAGAGATTTGCTCCCTGGAGTC and *Dyn* reverse primer: CCAAGCCTTCTAATACGACTCACTATAGGGAGACATCTCGGAACTCCTCTTGG and transcribed for ISH.

In situ *hybridization.* Antisense mouse *Kiss1* probe was transcribed from linearized pAMP1 plasmid containing the mouse *Kiss1* insert with T7 polymerase (Fermentas) (Gottsch et al., 2004), and *Dyn* sense and antisense probes were transcribed with T7 or T3 polymerase (Fermentas). Radiolabeled probes were synthesized *in vitro* by inclusion of the following ingredients in a volume of 20 μl: 250 μCi ^{33}P-UTP (Perkin-Elmer Life Sciences); 1 μg of linearized DNA (or 1 μg of PCR product); 0.5 mM each ATP, CTP, GTP; and 40 U of polymerase. Residual DNA was digested with 4 U of DNase (Ambion), and the DNase reaction was terminated by addition of 2 μl of 0.5 M EDTA, pH 8.0. The riboprobes were separated from unincorporated nucleotides with NucAway Spin Columns (Ambion).

Slides with mouse hypothalamic sections from the three experimental groups were processed before hybridization, as previously reported (Cunningham et al., 2002; Gottsch et al., 2004). Briefly, sections were fixed in 4% paraformaldehyde, pretreated with acetic anhydride, rinsed in 2× sodium citrate, sodium chloride (SSC), delipidated in chloroform, dehydrated in graded ethanols, and then allowed to air dry before the hybridization procedure. Radiolabeled antisense *Kiss1* riboprobes or antisense and sense *Dyn* riboprobes were denatured, dissolved in hybridization solution at a concentration of 0.1 pmol/ml along with tRNA (1.9 mg/ml) (Roche Biochemicals) in 0.1 M Tris/0.01 M EDTA, pH 8.0, to produce the probe mix. The probe mix was heat denatured in boiling water for 3 min, then returned to ice for 5 min. The denatured probe mix was added to prewarmed hybridization buffer (60% deionized formamide, 5× hybridization salts, 0.1× Denhardt's buffer, 0.2% SDS), at a ratio of 1:4, and added to each slide (100 μl/slide). The sections were then coverslipped and placed in humidity chambers at 55°C for 16 h. After hybridization, coverslips were removed, and the slides were washed in 4× SSC at room temperature. Slides were then placed into ribonuclease (RNase) [10 mg/ml RNase (Roche Biochemicals) in 0.15 M sodium chloride, 10 mM Tris, 1 mM EDTA, pH 8.0] for 30 min at 37°C, then in RNase buffer, without RNase, at 37°C for another 30 min. After a 30 min wash in 2× SSC at room temperature, slides were washed twice in 0.1× SSC at 62°C, then dehydrated in graded ethanols and air dried. The slides were then dipped in Kodak NTB emulsion (VWR), air dried, and stored at 4°C for 8–9 d. Slides were then developed, dehydrated in graded ethanols, cleared in Citrasol (VWR), and coverslips were applied with Permaslip (Sigma).

Kiss1 *and* Dyn *mRNA quantification and analysis.* All *Kiss1* or *Dyn* mRNA-containing sections were analyzed unilaterally. Slides from all of the animals were assigned a random three-letter code, alphabetized, and read under dark-field illumination with custom-designed software designed to count the total number of cells and the number of silver grains (corresponding to radiolabeled *Kiss1* or *Dyn* mRNA) over each cell (Chowen et al., 1990). The system consists of a Scion VG5 video acquisition board (Perceptics) attached to a Power Macintosh G5 computer running custom grain-counting software. Cells were counted as *Kiss1* or *Dyn* mRNA positive when the number of silver grains in a cluster exceeded that of background. Thus, cell counts represent the number of cells that achieved a detectability threshold, and the grains per *Kiss1* or

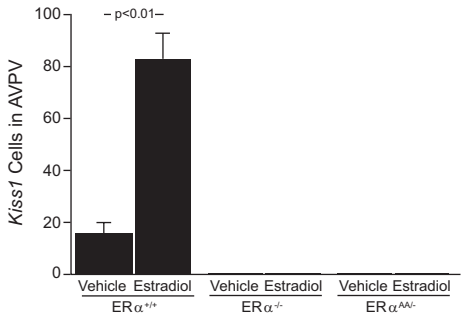

Figure 1. The effects of estradiol on the relative number of cells expressing *Kiss1* mRNA in the AVPV of female ERα$^{+/+}$, ERα$^{-/-}$, or ERα$^{AA/-}$ mice. There were 5–6 animals per group; bars represent means + SEM.

Dyn cell reflects a semiquantitative index of mRNA content in those cells that achieve the detectability threshold (Cunningham et al., 2002; Gottsch et al., 2004; Smith et al., 2005). Cells were considered *Kiss1* or *Dyn* positive when the number of silver grains in a cluster exceeded that of background by threefold.

Statistical analysis. All data are expressed as the mean ± SEM for each group. ANOVA followed with Tukey/Kramer *post hoc* tests were used to assess variation among experimental groups in each experiment. Significance level was set at $p < 0.05$. All analyses were performed with Statview 5.0.1 for Macintosh (SAS Institute).

Results

Serum hormone levels

Serum levels of LH in ERα$^{+/+}$ female OVX + vehicle-treated mice were elevated relative to those of OVX + E_2-treated ERα$^{+/+}$ mice. Treatment of ERα$^{+/+}$ OVX female mice adults with E_2-filled implants significantly reduced LH concentrations (ERα$^{+/+}$ OVX + vehicle = 2.88 ± 0.55 ng/ml vs ERα$^{+/+}$ OVX + E_2 = 0.23 ± 0.03 ng/ml; $p < 0.01$), indicating that the implants were functional and able to provide steroidal negative feedback. The LH values of ERα$^{-/-}$ mice were elevated regardless of treatment (ERα$^{-/-}$ OVX + vehicle mice = 3.60 ± 1.05 ng/ml vs ERα$^{-/-}$ OVX + E_2 = 1.63 ± 0.27 ng/ml; $p = 0.08$), and the LH values of OVX + E_2-treated ERα$^{AA/-}$ mice were decreased compared with OVX + vehicle-treated ERα$^{AA/-}$ mice (ERα$^{AA/-}$ OVX + vehicle = 5.90 ± 1.13 ng/ml vs ERα$^{AA/-}$ OVX + E_2 = 0.46 ng/ml ± 0.27 ng/ml; $p < 0.01$).

E_2 regulation of *Kiss1* mRNA in the AVPV

In the AVPV, the results observed in ERα$^{+/+}$ mice were the opposite of those found in the Arc of ERα$^{+/+}$ mice. In ERα$^{+/+}$ that were OVX + vehicle replaced, the number of identifiable *Kiss1* mRNA-containing cells was decreased compared with those ERα$^{+/+}$ mice that were OVX + E_2 replaced (ERα$^{+/+}$ OVX + vehicle mice = 17.5 cells vs ERα$^{+/+}$ OVX + E_2 mice = 83 ± 11 cells; $p < 0.01$) (Figs. 1, 2), as was the cellular content of *Kiss1* mRNA in OVX + vehicle-treated ERα$^{+/+}$ mice [ERα$^{+/+}$ OVX + vehicle mice = 39.5 ± 3.5 grains per cell (gpc) vs ERα$^{+/+}$ OVX + E_2 mice = 114.8 ± 27.3 gpc; $p < 0.01$; data not shown]. In the AVPV of ERα$^{-/-}$ mice, the number of *Kiss1* mRNA-expressing cells was not changed with OVX + vehicle treatment or OVX + E_2 treatment, as there were essentially no identifiable cells in either group of ERα$^{-/-}$ mice (Fig. 1). The same was true for ERα$^{AA/-}$ mice, in which there were no identifiable *Kiss1* cells in the AVPV regardless of treatment (Figs. 1, 2).

E_2 regulation of *Kiss1* mRNA expression in the Arc

In the Arc of vehicle-treated ERα$^{+/+}$ mice, OVX increased the number of cells expressing *Kiss1* mRNA (OVX + vehicle = 91 ± 16 cells vs no identifiable *Kiss1* cells in E_2-treated ERα$^{+/+}$ mice; $p < 0.01$) (Figs. 3, 4) and increased the cellular content of *Kiss1* mRNA as reflected by gpc (140 ± 7 gpc vs no gpc in E_2-replaced ERα$^{+/+}$ mice; $p < 0.01$; data not shown). In ERα$^{-/-}$ mice, there was no effect of E_2 upon the number of *Kiss1* mRNA-expressing cells in the Arc ($p > 0.05$; OVX + vehicle ERα$^{-/-}$ mice = 43.4 ± 17.4 cells vs OVX + E_2 ERα$^{-/-}$ mice = 47 ± 15 cells) (Figs. 3, 4). Nor was there any effect of treatment upon *Kiss1* mRNA content in these cells (ERα$^{-/-}$ OVX + vehicle mice = 157 ± 33 gpc vs ERα$^{-/-}$ OVX + E_2 mice = 99 ± 14 gpc; $p > 0.05$; data not shown). In vehicle-treated ERα$^{AA/-}$ mice, the number of *Kiss1* mRNA-expressing cells was upregulated in the Arc compared with E_2-treated ERα$^{AA/-}$ mice (ERα$^{AA/-}$ OVX + vehicle mice = 101.3 ± 27.5 cells vs ERα$^{AA/-}$ OVX + E_2 mice = 20 ± 20 cells; $p < 0.05$) (Figs. 3, 4), and the cellular content of *Kiss1* mRNA was also increased (ERα$^{AA/-}$ OVX + vehicle mice = 130.5 ± 17.7 gpc vs ERα$^{AA/-}$ OVX + E_2 mice = 18.5 ± 18.5 gpc; $p < 0.01$; data not shown).

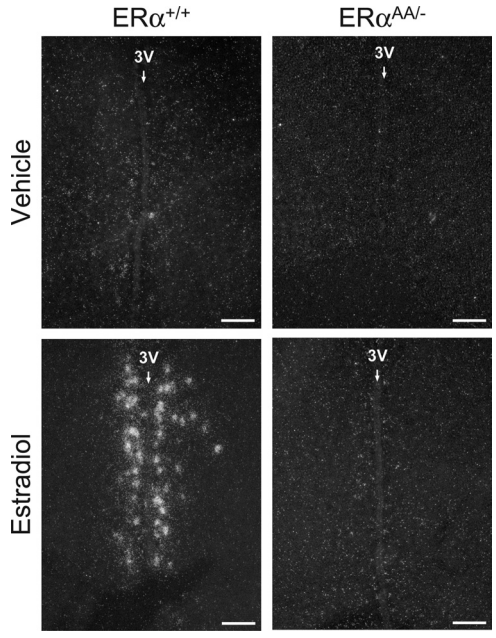

Figure 2. Photomicrographs depicting the effects of estradiol on *Kiss1* mRNA expression in the AVPV of female ERα$^{+/+}$ and ERα$^{AA/-}$ mice. Silver grains (white dots) represent *Kiss1* mRNA. Photomicrographs depicting *Kiss1* mRNA expression in ERα$^{-/-}$ mice were omitted, as they were the same as ERα$^{AA/-}$ mice. 3V, Third ventricle. Scale bars, 100 μm.

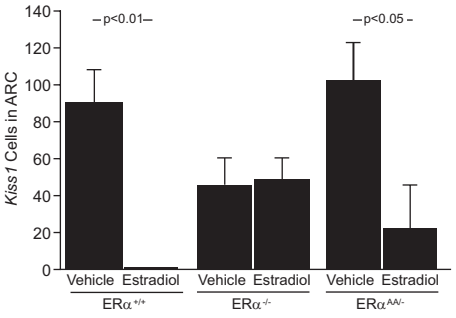

Figure 3. The effects of estradiol on the relative number of cells expressing *Kiss1* mRNA in the Arc of female ERα$^{+/+}$, ERα$^{-/-}$, or ERα$^{AA/-}$ mice. There were 5–6 animals per group; bars represent means + SEM.

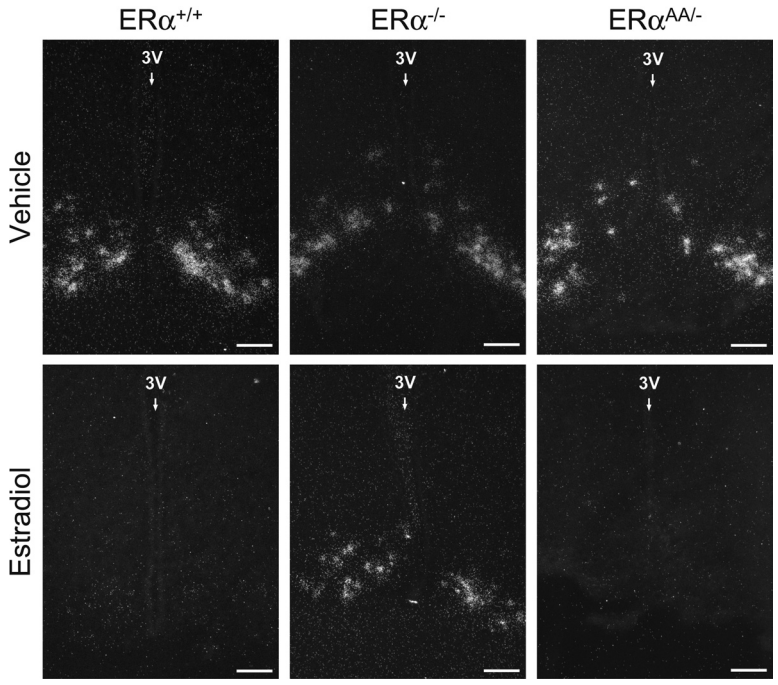

Figure 4. Photomicrographs depicting the effects of estradiol on *Kiss1* mRNA expression in the Arc of female ERα$^{+/+}$, ERα$^{-/-}$, and ERα$^{AA/-}$ mice. Silver grains (white dots) represent *Kiss1* mRNA. 3V, Third ventricle. Scale bars, 100 μm.

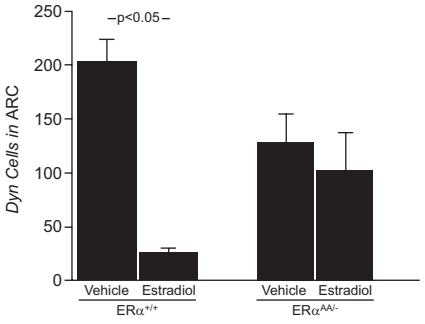

Figure 5. The effects of estradiol on the relative number of cells expressing *Dyn* mRNA in the Arc of female ERα$^{+/+}$ and ERα$^{AA/-}$ mice. There were 5–6 animals per group; bars represent means + SEM.

E_2 regulation of Dynorphin mRNA expression in the Arc

In the Arc of ERα$^{+/+}$ mice, OVX + vehicle treatment increased the number of cells expressing *Dyn* mRNA, as well as the cellular content of *Dyn* mRNA, compared with ERα$^{+/+}$ mice that were OVX + E_2 treated (ERα$^{+/+}$ OVX + vehicle mice = 197.6 ± 24.5 cells vs ERα$^{+/+}$ OVX + E_2 mice = 25.3 ± 6.15 cells; $p < 0.05$) (Figs. 5, 6) (ERα$^{+/+}$ OVX + vehicle mice = 57.9 ± 8.1 gpc vs ERα$^{+/+}$ OVX + E_2 mice = 23.6 ± 3.9 gpc; $p < 0.05$; data not shown). In vehicle-treated ERα$^{AA/-}$ mice, the number of *Dyn* mRNA-expressing cells was not changed in the Arc compared with E_2-treated ERα$^{AA/-}$ mice (ERα$^{AA/-}$ vehicle-treated mice = 128.2 ± 28 cells vs ERα$^{AA/-}$ E_2-treated mice = 103.2 ± 35.6 cells; $p > 0.05$) (Figs. 5, 6), nor was the cellular content of *Dyn* mRNA (ERα$^{AA/-}$ vehicle-treated mice = 46.5 ± 8.6 gpc vs ERα$^{AA/-}$ E_2-treated mice = 35.7 ± 5.2 cells; $p > 0.05$; data not shown). In OVX vehicle-treated and OVX E_2-treated ERα$^{-/-}$ female mice, there was no change in *Dyn* expression (Navarro, Clifton, Steiner, unpublished observations). There were no identifiable *Dyn* cells on slides that received *Dyn* sense riboprobe (data not shown).

Discussion

The positive feedback effects of E_2 on LH release in the rodent have been extensively investigated over the past 60 years. It is now known that E_2 acts via ERα to induce the preovulatory GnRH/LH surge (Wintermantel et al., 2006), and that this process involves classical ERE-dependent signaling (Glidewell-Kenney et al., 2007). Since *Kiss1* mRNA expression is induced by E_2 in the AVPV—an area thought to be responsible for the generation of the preovulatory LH surge in rodents—we and others have argued that kisspeptin neurons in the AVPV participate in the E_2-induced preovulatory surge of GnRH/LH, i.e., reflecting the positive feedback effects of E_2 (Smith et al., 2005; Clarkson et al., 2008). If this were true, we would expect that E_2 would act through the same pathway to regulate *Kiss1* in the AVPV as it does to generate the GnRH/LH surge. We previously reported that E_2 acts via ERα in the AVPV to regulate *Kiss1* in mice and have confirmed that observation here (Smith et al., 2005). Furthermore, our current observations suggest that E_2 acts via a classical ERE-dependent pathway to regulate *Kiss1* in the AVPV, the same mechanism by which E_2 has been implicated to generate LH surges in mice (Glidewell-Kenney et al., 2007). Thus, our current findings are consistent with the hypothesis that kisspeptin neurons in the AVPV mediate the positive feedback effects of E_2 on GnRH/LH secretion.

Our observation that E_2 inhibits *Kiss1* in the Arc of mice with a mutated ERα that cannot bind to ERE sites (ERα$^{AA/-}$ mice) suggests that regulation of the *Kiss1* gene by E_2 in the Arc involves an ERE-independent signaling pathway. In a study of ERα$^{AA/-}$ mice, Glidewell-Kenney et al. (2007) previously demonstrated that negative feedback regulation of LH by E_2 is mediated by an ERE-independent mechanism. The notion that both GnRH/LH secretion and *Kiss1* gene expression in the Arc may be regulated by similar (or identical) ERα signaling pathways is consistent with the proposition that these *Kiss1* neurons participate in the negative feedback regulation of gonadotropin secretion. Although it would appear that the regulation of *Kiss1* by E_2 in the Arc of ERα$^{AA/-}$ mice is remarkably similar to that found in ERα$^{+/+}$, we cannot rule out some minor (but consequential) effects of classical ERα signaling, ERβ signaling, or other nonclassical ER signaling pathways.

E_2 can act through ERα to regulate gene expression in an ERE-independent manner in several ways. E_2 bound to ERα may also interact with AP-1, Sp1, or NF-κB, which in turn can associate with target DNA elements to induce transcriptional activation (Gaub et al., 1990; Ray et al., 1994; Webb et al., 1995; Kushner et al., 2000; Safe, 2001; Jakacka et al., 2002; Khan et al., 2003; Safe and Kim, 2004; Fleming et al., 2006). The *Kiss1* promoter contains both AP-1 and Sp1 sites, and *in vitro* studies examining the *Kiss1* promoter have revealed that Sp1 elements within the first 200 bp of the *Kiss1* promoter can regulate *Kiss1* gene expression in certain cell lines (Li et al., 2007; Mitchell et al., 2007). Whether this is the case for the *in vivo* regulation of *Kiss1* by E_2 in the Arc of the female mouse remains to be determined. However, in this case we

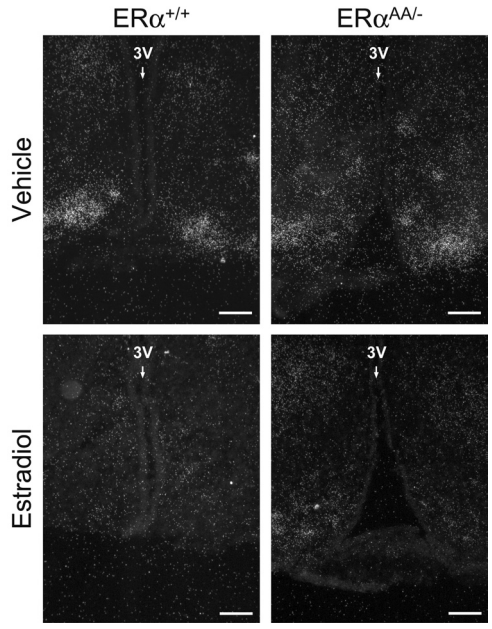

Figure 6. Photomicrographs depicting the effects of estradiol on *Dyn* mRNA expression in the Arc of female ERα$^{+/+}$ and ERα$^{AA/-}$ mice. Silver grains (white dots) represent *Dyn* mRNA. 3V, Third ventricle. Scale bar, 50 μm.

have shown that nonclassical ERα signaling pathways are sufficient to regulate *Kiss1* gene expression in the Arc in the absence of ERE-dependent ERα signaling pathways. The molecular elements that signal through this nonclassical pathway to regulate *Kiss1* expression in the Arc have yet to be identified.

Dyn is coexpressed with *Kiss1* in the Arc of mice (Navarro, Clilfton, Steiner, unpublished observations), as has been previously shown in the sheep by Goodman et al. (2007). We were surprised to find that E_2 regulates *Dyn* mRNA through ERE-dependent ERα signaling, whereas in the same cells, *Kiss1* is regulated in an ERE-independent manner. Whether this regulation of *Dyn* and *Kiss1* expression by two different ER signaling pathways occurs in all species is unknown. However, the observations reported here imply that ERα can regulate gene expression within individual neurons through multiple pathways in the same cell population. Although genes within the same neuron may be regulated in a parallel manner by E_2 acting through the same ER subtype (i.e., either induced or suppressed), different molecular pathways may be involved in the regulation of each gene. Furthermore, since E_2 regulates the *Dyn* gene in an ERE-dependent manner, its regulation does not appear to play an important role in the negative feedback control of LH secretion. This is because E_2 effectively suppresses LH secretion in animals with a mutated ERα allele that cannot bind ERE sites, yet it does not change *Dyn* gene expression (cf. Glidewell-Kenney et al., 2008). Thus, despite the compelling case for kisspeptin in the Arc mediating the negative feedback effects of E_2 on GnRH/LH secretion, the function of dynorphin in these same *Kiss1* neurons remains a mystery.

There is some concern that the ERα$^{AA/-}$ mouse model might mask a truly ERE-independent signaling mechanism. For example, if the development of *Kiss1* neurons in the AVPV required an intact ERE-dependent ERα signaling pathway, there would be no *Kiss1* neurons in the AVPV that respond to E_2 in ERα$^{AA/-}$ mice. If this were the case, even if an ERE-independent pathway normally regulated the expression of *Kiss1*, E_2 may be incapable of inducing *Kiss1* in such an animal. Our observation that the AVPV of ERα$^{-/-}$ and ERα$^{AA/-}$ is virtually devoid of *Kiss1* neurons is consistent with this alternative hypothesis. Indeed, this is not what one would expect, since the congenital lack of ERα signaling should in theory yield a feminized AVPV (Bodo et al., 2006; McDevitt et al., 2008). Thus, it is plausible that the presence of an ERE-dependent ERα signaling pathway is a prerequisite for the appropriate development of *Kiss1* neurons in the AVPV of females. Another problem with interpreting the finding in the ERα$^{AA/-}$ mouse is that it should not be assumed that only a single pathway is involved. For example, if ERE-dependent and -independent mechanisms were required to produce an effect, the ERα$^{AA/-}$ results would be interpreted to indicate that only an ERE-dependent mechanism was involved. This would not be a major difficulty if both pathways worked in parallel to produce an effect, which would result in a blockade of the effect in the ERα$^{-/-}$ mice and an attenuated response in the ERα$^{AA/-}$ mice. A recent report suggests that this may be the case with respect to negative feedback regulation of LH, where E_2 had no effect on LH in ERα$^{-/-}$ mice, and was ~70% as effective in ERα$^{AA/-}$ mice compared with ERα$^{+/+}$ (Glidewell-Kenney et al., 2007). We observed a similar trend in the regulation of *Kiss1* expression in the Arc, although the differences in the effect of E_2 on levels *Kiss1* mRNA between ERα$^{+/+}$ and ERα$^{AA/-}$ mice were not statistically significant. Despite these limitations and concerns, the ERα$^{AA/-}$ mouse can be a useful tool to help unravel the pathways involved in ERα-mediated signaling.

In summary, we have used the ERα$^{AA/-}$ mouse model to demonstrate that the inhibition of *Kiss1* gene expression by E_2 in the Arc appears to be mediated by an ERE-independent ERα signaling mechanism, whereas the inhibition of *Dyn* expression in the Arc and the induction of *Kiss1* in the AVPV is likely to occur through ERE-dependent ERα signaling pathways. Our results investigating the regulation of *Kiss1* by ERα parallel an earlier report demonstrating that negative feedback regulation of LH is mediated by ERE-independent mechanisms, whereas ERE-dependent ERα signaling mechanisms mediate the positive feedback regulation of LH and ovulation. These observations provide additional evidence that *Kiss1* neurons in the Arc participate in negative feedback regulation of GnRH/LH secretion by E_2 and that *Kiss1* neurons in the AVPV are involved in the positive feedback action of E_2 that leads to the generation of the preovulatory GnRH/LH surge.

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