Estrogen Stimulation of *Kiss1* Expression in the Medial Amygdala Involves Estrogen Receptor-α But Not Estrogen Receptor-β

Shannon B. Z. Stephens, Navdeep Chahal, Nagambika Munaganuru, Ruby A. Parra, and Alexander S. Kauffman

Department of Reproductive Medicine, University of California, San Diego, La Jolla, California 92093

The neuropeptide kisspeptin, encoded by *Kiss1*, regulates reproduction by stimulating GnRH secretion. Neurons synthesizing kisspeptin are predominantly located in the hypothalamic anteroventral periventricular (AVPV) and arcuate nuclei, but smaller kisspeptin neuronal populations also reside in extrahypothalamic brain regions, such as the medial amygdala (MeA). In adult rodents, estradiol (E_2) increases *Kiss1* expression in the MeA, as in the AVPV. However, unlike AVPV and arcuate nuclei kisspeptin neurons, little else is currently known about the development, regulation, and function of MeA *Kiss1* neurons. We first assessed the developmental onset of MeA *Kiss1* expression in males and found that MeA *Kiss1* expression is absent at juvenile ages but significantly increases during the late pubertal period, around postnatal day 35, coincident with increases in circulating sex steroids. We next tested whether developmental MeA *Kiss1* expression could be induced early by E_2 exposure prior to puberty. We found that juvenile mice given short-term E_2 had greatly increased MeA *Kiss1* expression at postnatal day 18. Although MeA *Kiss1* neurons are known to be E_2 up-regulated, the specific estrogen receptor (ER) pathway(s) mediating this stimulation are unknown. Using adult ERα knockout and ERβ knockout mice, we next determined that ERα, but not ERβ, is required for maximal E_2-induced MeA *Kiss1* expression in both sexes. These results delineate both the developmental time course of MeA *Kiss1* expression and the specific ER signaling pathway required for E_2-induced up-regulation of *Kiss1* in this extrahypothalamic brain region. These findings will help drive future studies ascertaining the potential functions of this understudied kisspeptin population. **(*Endocrinology* 157: 4021–4031, 2016)**

Kisspeptin, a neuropeptide encoded by the *Kiss1* gene, is essential for reproduction, as demonstrated by the fact that humans and mice harboring mutations in the *Kiss1r* or *Kiss1* genes show striking deficits in puberty, reproductive hormone release, and fertility (1–4). *Kiss1* neurons are located primarily in the hypothalamic anteroventral periventricular (AVPV) and arcuate (ARC) nuclei, but a smaller population of *Kiss1* neurons is also present outside the hypothalamus in the medial amygdala (MeA) (5–12). Most kisspeptin research has focused on the regulation and reproductive role of the two hypothalamic *Kiss1* populations, and very little is currently known about the development, regulation, and function of MeA *Kiss1* neurons (8, 10, 11, 13). The MeA has numerous behavioral and physiological functions, including but not limited to effects on reproductive physiology and behavior (14–19). Thus, understanding the developmental and regulatory aspects of MeA *Kiss1* neurons may provide important insight into potential functions of kisspeptin signaling arising from the MeA.

Kiss1 gene expression in the two hypothalamic regions is differentially regulated by sex steroids (T and estradiol [E_2]) (8–10), and these differential effects most likely reflect the different roles of these two *Kiss1* populations in positive and negative feedback. The ARC is likely involved in mediating negative feedback of sex steroids because

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in USA

Received June 16, 2016. Accepted August 12, 2016.
First Published Online August 26, 2016

Abbreviations: ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus; E_2, estradiol; ER, estrogen receptor; GDX, gonadectomized; GPR, G protein-coupled receptor; ISH, in situ hybridization; KO, knockout; MeA, medial amygdala; PND, postnatal day; RNase, ribonuclease; SSC, sodium citrate/sodium chloride; WT, wild type.

removal of sex steroids via gonadectomy greatly increases ARC *Kiss1* expression, and exogenous T or E_2 treatment suppresses ARC *Kiss1* levels (8–10, 20). In contrast to the ARC, in the AVPV, gonadectomy significantly decreases *Kiss1* expression, whereas exogenous E_2 treatment robustly increases AVPV *Kiss1* expression, potentially linking the AVPV kisspeptin population to a role in E_2-positive feedback in females (8–10). As in the AVPV, we have previously reported that *Kiss1* expression in the MeA is greatly reduced after gonadectomy (11). Moreover, like in the AVPV, treatment with either T or E_2, but not DHT, upregulates *Kiss1* levels in the MeA (11). The lack of DHT effect indicates that sex steroid stimulation of *Kiss1* in the MeA occurs specifically through estrogen receptor (ER)-mediated pathways, rather than androgen receptor pathways (11). However, exactly which specific ER pathway is responsible for mediating E_2's up-regulation of *Kiss1* in the MeA is not currently known. Previous studies indicate that E_2 regulation of ARC and AVPV *Kiss1* occurs primarily via ERα (8, 10, 21). However, unlike the ARC and AVPV, which both express ERα at much higher levels than ERβ (10), the MeA highly expresses both ERα and ERβ (22, 23), suggesting either (or both) ERs may regulate MeA *Kiss1* expression.

We previously demonstrated that *Kiss1* is expressed in the MeA of adults (11) but is not readily detectable at postnatal day (PND) 14 in juvenile mice (24). Likewise, MeA *Kiss1* is not readily detectable at the prepubertal age of PND 19 in rats (25). This is in stark contrast to *Kiss1* in the hypothalamus, which is readily expressed in the ARC before birth and in the AVPV starting around the second to third week of postnatal life (26–29). It is currently unknown when *Kiss1* first becomes expressed in the MeA or whether the developmental onset of MeA *Kiss1* expression relates to pubertal increases in gonadal steroids because MeA *Kiss1* is up-regulated by sex steroids.

This study had several main goals to fill in gaps in our knowledge about the development and regulation of MeA *Kiss1* neurons. First, we examined the developmental expression of MeA *Kiss1* in juvenile, prepubertal, and peripubertal males to determine when MeA *Kiss1* is first expressed, how this expression changes with pubertal development, and whether any changes in *Kiss1* expression coincide with pubertal changes in circulating sex steroid levels. Next, we determined whether MeA *Kiss1* expression in juvenile male mice could be enhanced after exogenous short-term E_2 treatment, thereby assessing whether MeA *Kiss1* expression could be developmentally induced by early sex steroid exposure or whether the developmental onset of MeA *Kiss1* expression is guided by nonsex steroid factors. In the final set of experiments, we used adult ERα knockout (KO) and ERβKO mice of both sexes to determine which specific ER, ERα, and/or ERβ (both of which are very highly expressed in the MeA), is necessary for E_2's ability to up-regulate *Kiss1* in the MeA.

Materials and Methods

Animals

Experiments used either male C57BL6 mice (experiments 1 and 2) or mice of both sexes from the ERαKO or ERβKO lines (experiments 3 and 4). Male and female ERαKO mice and WT littermate controls were generated from heterozygous breeder ERαKO mice (obtained from Dr James L. Jameson, Philadelphia, Pennsylvania, and originally created by the Chambon laboratory) (30). Male and female ERβKO and WT control mice were similarly generated from heterozygous ERβKO breeders (Jackson Labs). All experimental animals were housed in a 12-hour light, 12-hour dark cycle at approximately 22°C. Mice were weaned at 21 days of age and housed two to three mice/cage (mixed WTs and KOs), unless noted otherwise, with ad libitum access to food and water. All surgeries occurred under isoflurane anesthesia. All experimental procedures were approved by the University of California, San Diego, Institutional Animal Care and Use Committee in accordance with the National Institute of Health policies.

Tissue collection

All blood sampling and tissue collection was performed on mice that were briefly anesthetized with isoflurane and then rapidly decapitated. Blood samples were collected retroorbitally just prior to decapitation and centrifuged (15 min at 5000 rpm) 90 minutes after collection and the serum stored at −20°C. Serum samples were assayed in singlet by the University of Virginia's Center for Research in Reproduction Ligand Assay and Analysis Core to measure the LH, E_2, and/or T levels. Brains were collected immediately after decapitation, frozen on dry ice, and stored at −80°C until sectioning. Brains were sectioned on a cryostat into five coronal sets of 20 μm sections spanning the entire hypothalamus and amygdala regions. Brain slices were mounted on to SuperFrost Plus slides (VWR Scientific) and stored at −80°C until in situ hybridization (ISH).

Single-label ISH

Single-label ISH for *Kiss1* expression was performed as previously described (26, 31–35). Briefly, one of the five coronal sets of slides containing the entire AVPV (experiments 2–4) or ARC and MeA (experiments 1–4) was assayed to examine *Kiss1* expression. Slides were first fixed in 4% paraformaldehyde and then pretreated with acetic anhydride and rinsed in a 2× sodium citrate/sodium chloride (SSC) solution. Slides were then delipidated in chloroform, dehydrated in ethanol washes, and air dried for 90 minutes. Radiolabeled (^{33}P) *Kiss1* (0.04 pmol/mL) antisense riboprobes were added to tRNA, heat denatured, and combined with hybridization buffer, and then 100 μL of this probe mixture was applied to each slide before hybridizing overnight in a 55°C humidity chamber. The next day, slides were washed in 4× SSC at room temperature and treated with ribonuclease (RNase) A for 30 minutes at 37°C. Slides were then washed in RNase buffer (no RNase A) at 37°C and 2× SSC at room tem-

perature, each for 30 minutes. After washes in 0.1× SSC at 62°C for 1 hour, the slides were dehydrated in ethanols, air dried for 90 minutes, dipped in Kodak nitroblue tetrazolium salt emulsion, air dried for 90 minutes, and then stored at 4°C until being developed. AVPV slides were developed 3–5 days later, depending on the experiment, whereas ARC and MeA slides were developed 9–12 days later, depending on the experiment. Due to the large size of the number of slides, male and female assays were run separately for the ERαKO and ERβKO experiments.

As in previous studies (26, 31–34), we used an automated computer image processing system (Dr Don Clifton, University of Washington, Seattle, Washington) to quantify *Kiss1* expression for each brain slice. The custom software takes into account background staining levels and counts the number of silver grain clusters, representing cells expressing *Kiss1* mRNA. We analyzed both the number of *Kiss1* cells and grains per cell during data analysis. However, for most assays of the MeA and AVPV, there was at least one of the four treatment groups that had either no cells or very few *Kiss1* cells (eg, none to two cells). As such, the grains per cell for those groups would not be calculable or would be based on just one or two cells, essentially eliminating that group from any proper statistical analyses. Thus, we do not report data for grains per cell, but we note that the data for grains per cell showed a comparable pattern as was seen with the number of *Kiss1* cells. All ISH slides were analyzed by a person blinded to treatment groups.

Experiment 1: when are MeA Kiss1 neurons initially expressed in development and does this coincide with developmental increases in gonadal steroids?

Hypothalamic *Kiss1* expression has been the focus of most kisspeptin research, but very little is known about the regulation and development of *Kiss1* neurons in the MeA. Although MeA *Kiss1* expression is readily found in adult mice (8, 10, 11), it was not previously observed in juvenile (PND 14) mice (24), and at present, nothing is known about the developmental pattern of MeA *Kiss1* expression. Because MeA *Kiss1* neurons are strongly up-regulated by E_2 (11) and gonadal steroid levels are not elevated until puberty, we hypothesized that MeA *Kiss1* would first be detected around the age of puberty. In this experiment, we therefore examined the developmental time course of MeA *Kiss1* expression in male mice and assessed how that relates to pubertal changes in sex steroid levels. Because MeA *Kiss1* expression is notably greater in gonad-intact adult males than females, we therefore used males in this experiment to assess developmental MeA *Kiss1* expression and to ensure detection of MeA *Kiss1* at early ages in gonad-intact animals. Developing C57BL6 male mice were briefly anesthetized with isoflurane and killed via rapid decapitation at PND 15, 20, 25, 30, 35, or 40. In male mice, the first two ages are juvenile, the next two are prepubertal, PND 35 is peripubertal, and PND 40 is around the onset of adulthood. At the time the animals were killed, blood and brains were collected to analyze serum T levels and MeA *Kiss1* expression, respectively (n = 4–6 mice per group).

Experiment 2: is MeA *Kiss1* expression capable of being induced prior to puberty with exogenous E_2 treatment?

Experiment 1 determined that MeA *Kiss1* expression was essentially absent at juvenile and prepubertal ages but became progressively more abundant during the pubertal ages and beyond, correlating with simultaneous increases in circulating sex steroid levels. Because MeA *Kiss1* expression in adults is strongly up-regulated with E_2 (11), we hypothesized that MeA *Kiss1* expression is not observed at young juvenile/prepubertal ages because circulating gonadal steroids are too low. However, it is also possible that MeA *Kiss1* expression is not observed at juvenile ages because MeA *Kiss1* neurons are not fully developed at this age. In this experiment, we gave short-term exogenous E_2 capsules to PND 14 male mice to determine whether early sex steroid exposure could induce a precocious rise in the MeA *Kiss1* levels. Juvenile male C57BL6 mice (PND 14) received either a small SILASTIC brand capsule (Dow Corning) with 1 mm of 1:25 E_2 to cholesterol mix (based on doses used in experiments 3 and 4) or received no exogenous hormonal treatment. All mice were then killed 4 days later on PND 18, and brains were collected to measure *Kiss1* expression in the MeA (n = 8–10 mice per group).

Experiment 3: is ERβ necessary for the E_2-induced increase in *Kiss1* in the MeA?

It has been shown that short-term T or E_2 treatment, but not DHT, robustly increases *Kiss1* levels in the MeA of adult mice and rats of both sexes (11), but the specific ER pathway(s) mediating this stimulatory effect is currently unknown. ERα appears to be the key ER pathway for regulating hypothalamic *Kiss1* neurons (8, 10). However, unlike the *Kiss1* neurons in the hypothalamus, which expresses ERα at much higher levels than ERβ (10), the MeA contains very high concentrated levels of both ERβ and ERα (22, 23), indicating that either (or both) of these ER pathways might mediate the stimulatory effects of E_2 on MeA *Kiss1* neurons. In experiment 3, we used ERβKO mice (lacking ERβ globally) to determine whether functional ERβ is necessary for E_2-induced *Kiss1* expression in the MeA. Adult ERβKO mice of both sexes, and their WT littermates, were gonadectomized (GDX) at 7 weeks of age. One week after GDX, all mice were individually housed and received either a SILASTIC brand capsule (Dow Corning) containing 2 mm of a 1:25 mixture of E_2 to cholesterol (previously shown to provide constant high elevated E_2 levels in adult mice) or no hormonal treatment. Five days later, all mice were killed, and blood and brains were collected to measure hormone levels and to examine *Kiss1* expression in the MeA and hypothalamus (n = 5–8 mice per group).

Experiment 4: is ERα necessary for the E_2-induced increase in *Kiss1* in the MeA?

Expression of *Kiss1* mRNA in the MeA is robustly upregulated by E_2 (11), but the specific ER pathway mediating this increase in MeA *Kiss1* expression is unknown. ERα is the primary receptor regulating both AVPV and ARC *Kiss1* neurons (8, 10), and the MeA contains high levels of ERα (22, 23, 36). As a complementary experiment to experiment 3 above, experiment 4 used ERαKO mice (global ERα knockouts) to determine whether ERα is specifically required for the E_2 up-regulation of MeA *Kiss1*. As was done for ERβKO mice, ERαKO and WT mice of both sexes were GDX at 7 weeks of age, received no hormonal treatment or an exogenous E_2 SILASTIC brand capsule (Dow Corning) at 8 weeks of age, and the brains and blood were collected 5 days later (n = 5–10 mice per group).

Statistical analysis

All data are expressed as mean ± SEM. For experiment 1, MeA *Kiss1* expression in each developmental age group was compared with MeA *Kiss1* expression in the PND15 group using a one-way ANOVA and Bonferroni correction post hoc tests. When examining *Kiss1* expression in the AVPV and MeA (experiments 2–4), at least one of the treatment groups had low *Kiss1* expression (eg, < 10 cells) and one treatment group had much higher *Kiss1* expression (eg, ≥ 50 cells), so the variances were not equal between groups and required the appropriate use of Mann-Whitney *U* (experiment 2; AVPV analysis) or Kruskal-Wallis (experiments 3 and 4) tests, using Dunn's multiple comparison tests for post hoc analysis. In experiment 2, there were no *Kiss1* cells found in the MeA of non-E_2-treated PND18 mice, and thus, a one-sample Student's *t* test was used to determine whether the number of MeA *Kiss1* cells induced by E_2 treatment at PND 18 was significantly greater than 0. When examining ARC *Kiss1* expression (experiments 3 and 4), group differences were examined using ANOVAs and Bonferroni post hoc tests. Statistical significance was set at $P < .05$ for all experiments.

Results

Experiment 1: MeA *Kiss1* and T levels show a similar developmental increase around PND 35

This experiment examined MeA *Kiss1* expression in male mice during development to identify when MeA *Kiss1* is initially expressed, how this expression changes during puberty, and whether the expression coincides with developmental increases in gonadal T secretion. MeA *Kiss1* expression was virtually absent at juvenile ages PND 15 and 20 but then gradually increased throughout peripubertal development, with a significant increase in MeA *Kiss1* first observed at PND 35 ($P < .05$, relative to PND 15, Figure 1). The highest MeA *Kiss1* expression was detected at PND 40. Circulating T levels in the same developing males followed a similar pattern to that of MeA *Kiss1* expression, with T levels being low at juvenile and prepubertal ages and then significantly increasing at PND 35 and PND 40 ($P < .05$ relative to PND 15, Figure 1).

Experiment 2: juvenile E_2 treatment significantly increases MeA Kiss1 expression

The previous experiment showed that *Kiss1* expression in the MeA is absent or very low at juvenile and prepubertal ages when circulating sex steroids are also low but then starts to increase at pubertal ages in synchrony with increased sex steroid levels. This suggests that the absence of MeA *Kiss1* expression at young juvenile ages may reflect a lack of stimulatory sex steroid signaling. To test this possibility, juvenile males (PND 14) were treated short term with a high dose of E_2 to determine whether early sex steroid exposure could prematurely increase MeA *Kiss1* expression at a young age. As a control comparison, *Kiss1* levels were also measured in the AVPV (where *Kiss1* is similarly stimulated by E_2 exposure). Similar to what has previously been observed in adult mice, E_2 treatment significantly increased *Kiss1* in the AVPV in PND 18 males in comparison with nontreated males, indicating the early E_2 treatment is sufficient to induce *Kiss1* in the AVPV (Figure 2; $P < .05$). As in the AVPV, early E_2 treatment also induced significantly more *Kiss1* expression in the amygdala on PND 18 (Figure 2; $P < .05$). Of note, the

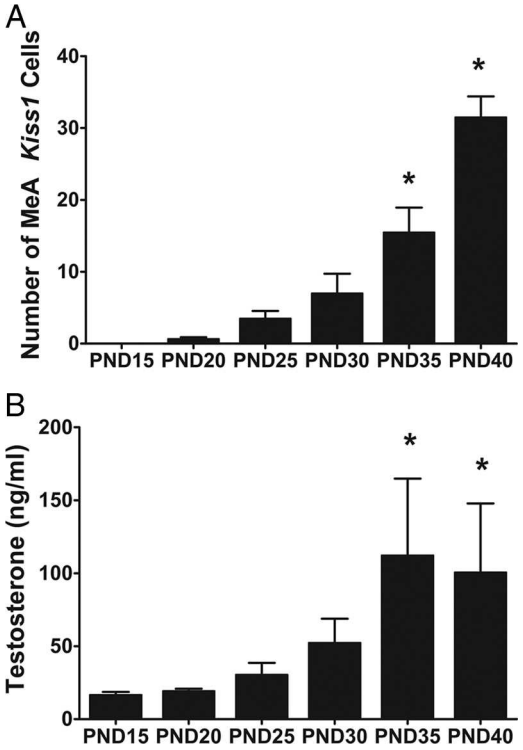

Figure 1. Developmental expression of MeA *Kiss1* in male mice. MeA *Kiss1* expression (A) and serum T (B) increase throughout development in male C57BL6 mice, with both measures demonstrating a significant increase around PND 35. *, $P < .05$, relative to PND 15.

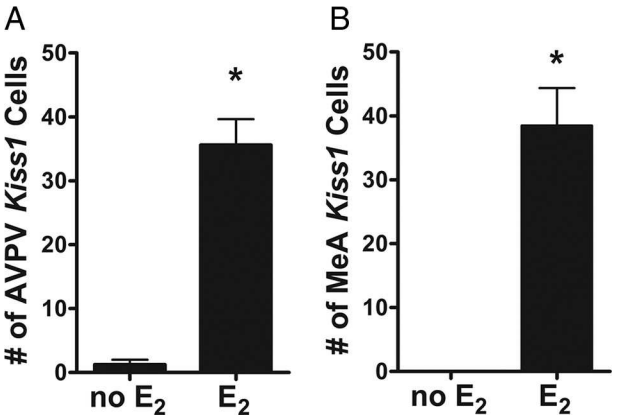

Figure 2. *Kiss1* mRNA expression in the AVPV (A) and MeA (B) of PND 18 male mice with or without short-term E_2 treatment. E_2 exposure significantly increased *Kiss1* levels in both AVPV and MeA of juvenile males, though *Kiss1* cells were more scattered throughout the MeA region than typically seen in adults. *, $P < .05$.

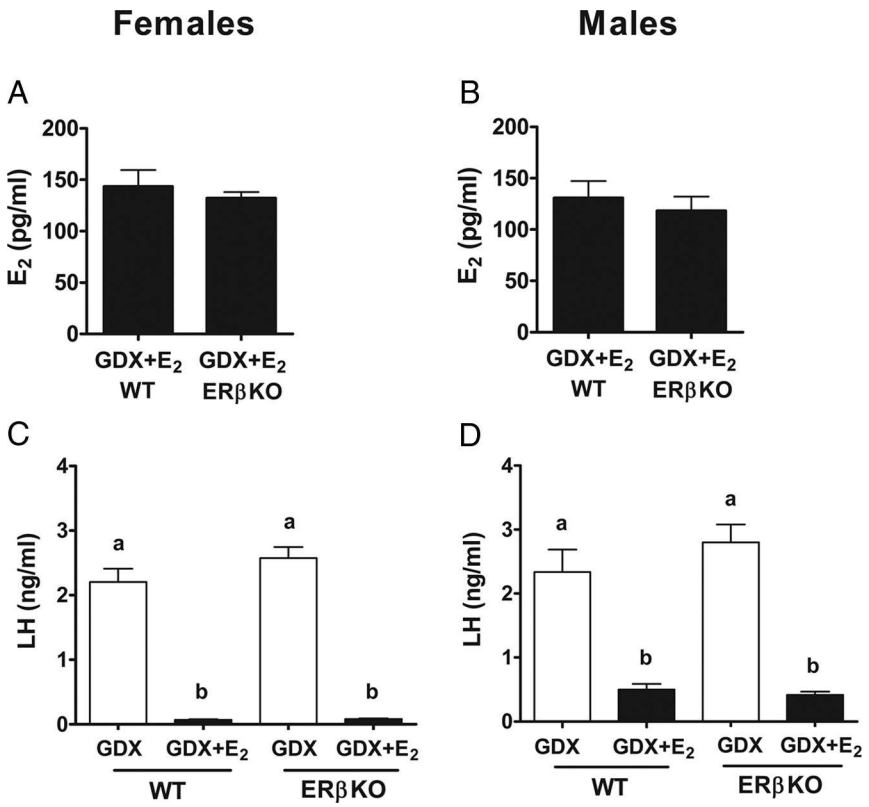

Figure 3. Serum E_2 and LH levels for male and female ERβKO mice with or without 5-day E_2 implants. E_2 levels were elevated in both WT and ERβKO E_2-treated female (A) and male (B) mice. LH levels were suppressed by E_2 in both WT and ERβKO female (C) and male (D) mice. Different letters denote significant group differences within each sex ($P < .05$).

heightened *Kiss1* expression in the amygdala at this age appeared more scattered throughout the greater MeA region than normally observed in adults, in which the predominance of *Kiss1* cells are in the posterior dorsal region of the MeA along with additional cells scattered in other MeA regions.

Experiment 3: ERβ is not required for maximal *Kiss1* expression in the MeA

Kiss1 in the MeA is markedly increased with short-term E_2 treatment in adults, but the ER pathway(s) regulating this increase are unknown. This experiment determined whether ERβ, which is highly expressed in the MeA, is required for E_2 up-regulation of MeA *Kiss1*. In adult ERβKO mice, serum E_2 levels were significantly increased with 5-day E_2 treatment, with no significant differences in E_2 levels between WT and ERβKO mice (Figure 3; $P < .05$). Likewise, serum levels of LH (which are typically reduced by exogenous E_2 negative feedback) were elevated in GDX mice of both genotypes and significantly suppressed with E_2 treatment in both WT and ERβKO mice of both sexes (Figure 3; $P < .05$).

Because *Kiss1* expression in the AVPV is up-regulated by E_2, AVPV *Kiss1* expression served as a positive control measure for the E_2 treatment. Both WT and ERβKO GDX mice of both sexes showed low levels of *Kiss1* in the AVPV, which was increased similarly in both genotypes with E_2 treatment (Figures 4 and 5; $P < .05$). In contrast to the AVPV, in the ARC, both WT and ERβKO GDX mice had significantly more *Kiss1* cells than non-E_2-treated mice (Figures 4 and 5; $P < .05$). As was seen in the AVPV, in the MeA, *Kiss1* expression significantly increased with E_2 treatment in both WT and ERβKO mice (Figures 4 and 5; $P < .05$). This increased MeA *Kiss1* expression was present in both male and female ERβKOs, demonstrating that ERβ is not required for the E_2 up-regulation of *Kiss1* in the MeA, similar to *Kiss1* in the AVPV.

Experiment 4: ERα is required for maximal *Kiss1* expression in the MeA

This experiment determined whether ERα, which is also highly expressed in the MeA, is required for E_2 induction of MeA *Kiss1* expression. In ERαKO and WT mice, circulating E_2 levels were significantly elevated in 5-day E_2-treated mice in comparison with GDX mice (Figure 6; $P < .05$) and were comparable between genotypes (Figure 6; $P < .05$). Serum LH levels were significantly elevated after GDX in both WT and ERαKO mice and were significantly suppressed by E_2 treatment in WTs but not in ERαKOs of either sex (as expected, given the known necessary role for ERα in E_2 negative feedback) (Figure 6; $P < .05$).

GDX WT and ERαKO mice both showed low AVPV *Kiss1* levels, and E_2 treatment significantly increased AVPV *Kiss1* expression in WTs but not ERαKOs of either sex (Figure 7 and Supplemental Figure 1; $P < .05$), indicating that ERα is required for the E_2 up-regulation of *Kiss1* in the AVPV, as previously reported for just female mice. In contrast to the AVPV, in the ARC, GDX WT mice of both sexes had significantly more *Kiss1* cells than E_2-treated WT mice (Figure 7 and Supplemental Figure 1; $P < .05$). However, in ERαKOs, there was no E_2-induced suppression of ARC *Kiss1*, with all ERαKO mice, regardless of treatment, showing high ARC *Kiss1* levels comparable with those in GDX WT mice (Figure 7 and Supplemental Figure 1; $P < .05$). In the MeA, WT mice of both sexes

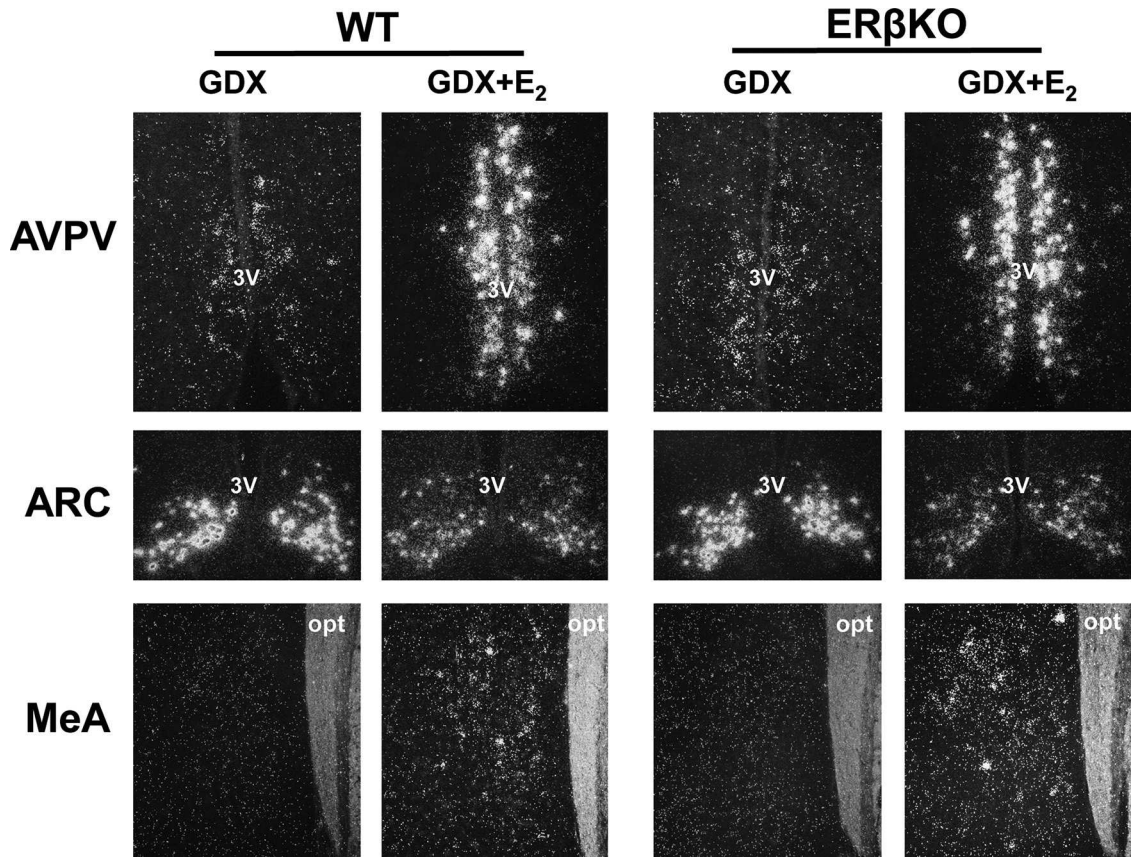

Figure 4. Representative images of *Kiss1* mRNA expression in the AVPV (female), ARC (male), and MeA (male) of ERβKO mice with or without 5-day E_2 treatment. opt, optic tract; 3V, third ventricle.

showed the expected E_2 up-regulation of *Kiss1* expression. Interestingly, both male and female ERαKO mice did exhibit a small but significant increase in MeA *Kiss1* levels with E_2 treatment relative to GDX ERαKOs; however, this E_2-induced increase in MeA *Kiss1* expression in ERαKO mice was of lower magnitude than the increase observed in their E_2-treated WT littermates (Figure 7 and Supplemental Figure 1; $P < .05$). This demonstrates that ERα is required for maximal MeA *Kiss1* expression, but another ER may also be involved in regulating MeA *Kiss1* to a lesser degree.

Discussion

Kisspeptin synthesized in the hypothalamus stimulates GnRH secretion and the reproductive axis (1, 5, 6, 37–41), and as a result, the vast majority of kisspeptin research has focused on the role of hypothalamic *Kiss1* neurons in reproduction and sex steroid feedback. However, *Kiss1* is also located at moderate levels in extrahypothalamic areas, such as the MeA (8, 10, 11, 24), a region of the limbic system involved in regulating numerous physiological and behavioral responses, including aspects of reproduction (14–19). Yet, at present, little is known about the development, regulation, and function of *Kiss1* neurons in the MeA. In the present study, we filled in several gaps in our knowledge about the development and regulation of MeA *Kiss1* neurons. First, we delineated the developmental time course of MeA *Kiss1* expression and linked this onset of expression, which occurs around puberty, to developmental sex steroid exposure. Next, using multiple global knockout mouse lines, we determined the specific ER signaling pathway that is required for E_2-induced up-regulation of *Kiss1* in this MeA region, identifying ERα, but not ERβ, as a critical mediator of E_2 signaling for MeA *Kiss1* stimulation.

Kiss1 is readily expressed in the MeA in adult rodents of both sexes (8, 10, 11, 24), but this is not the case at younger ages. Previous research demonstrated that, although *Kiss1* is already being expressed in the AVPV and ARC at young ages (26–29), *Kiss1* expression is absent in the MeA in juvenile (PND 14) mice (24) and prepubertal (PND 19) rats (25). However, it remained unknown when in development MeA *Kiss1* begins to be expressed between juvenile and adult stages. Moreover, given that MeA *Kiss1* is up-regulated by sex steroids (11), it was also unknown

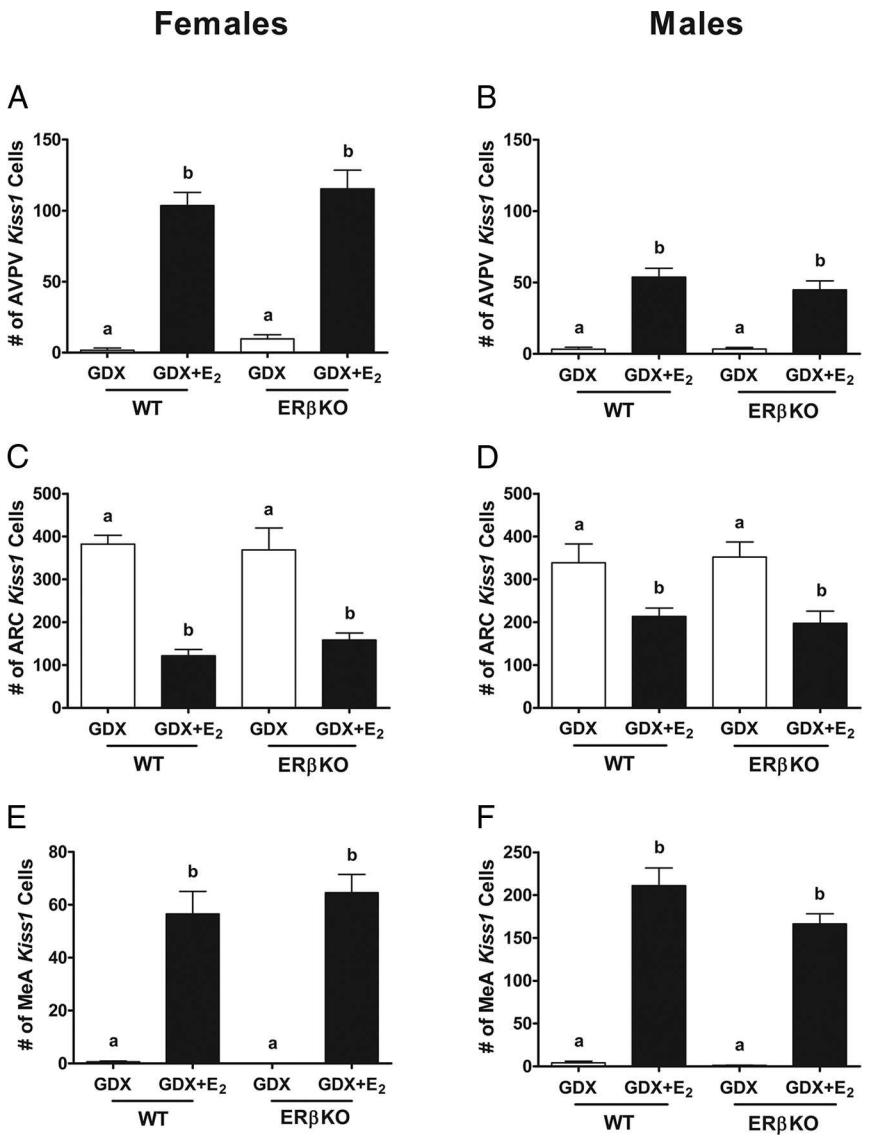

Figure 5. *Kiss1* mRNA expression in ERβKO mice. Mean number of *Kiss1* cells in the AVPV of female (A) and male (B), ARC of female (C) and male (D), and MeA of female (E) and male (F) ERβKO and WT littermates. Different letters denote significant group differences within each sex ($P < .05$).

how or whether this initial *Kiss1* expression related to developmental changes in circulating sex steroid hormones, which themselves increase at puberty. We therefore examined the developmental expression of *Kiss1* in the MeA in male mice every 5 days between PND 15 and PND 40. *Kiss1* mRNA in the MeA was essentially absent at both PND 15 and 20 (juvenile ages) but showed a small, gradual increase throughout prepubertal and peripubertal development, with a low number of cells present around PND 25–30 and then substantially more cells detected at subsequent later ages (PND 35 and 40). Interestingly, serum T levels mimicked a similar developmental pattern, with T levels being low initially and then gradually increasing but not showing a significant increase until PND 35 and 40. Given the synchrony in the developmental timing of the *Kiss1* and T increases, it suggests that MeA *Kiss1* expression onset is likely guided by gonadal sex steroid levels and first becomes notably expressed when T levels first begin to rise substantially around puberty.

Although MeA *Kiss1* neurons showed a significant increase in expression at PND 35, coinciding with an increase in serum T, this developmental pattern does not preclude the possibility that earlier *Kiss1* expression is absent because either *Kiss1* neurons are not fully developed yet or some additional stimulatory (or lessening of an inhibitory factor) is also required and not yet present. In experiment 2, we addressed this issue by testing whether MeA *Kiss1* expression could in fact be elicited earlier in development if higher circulating sex steroids were present. Indeed, as in adults, in juvenile (PND 18) male mice, short-term E_2 treatment significantly increased *Kiss1* levels in both the AVPV and the MeA in comparison with non-E_2-treated mice. This indicates that the lack of detectable MeA *Kiss1* expression at juvenile and prepubertal ages is most likely due to a lack of sufficient sex steroid exposure to stimulate the *Kiss1* gene at this time, rather than due to absent *Kiss1* neurons or an absent nonsteroidal stimulatory signal. Interestingly, we also note that *Kiss1* expression in these E_2-treated juveniles was less abundant in the posterodorsal subdivision of the MeA, in which *Kiss1* expression in adults is most predominant, but was observed more scattered throughout multiple parts of the greater medial amygdala area. It is not currently clear whether the abundant yet more scattered MeA *Kiss1* cells observed with E_2 treatment at PND 18 are the same cells expressing *Kiss1* in adulthood, which have migrated closer to the MePD subregion during puberty.

MeA *Kiss1* is up-regulated by E_2 (11), suggesting that a functional role of MeA *Kiss1* neurons involving kisspeptin signaling might occur primarily when sex steroid levels are elevated. However, many behaviors and physiological functions are modulated by either ERα or ERβ. Thus, understanding which specific ER(s) pathway regu-

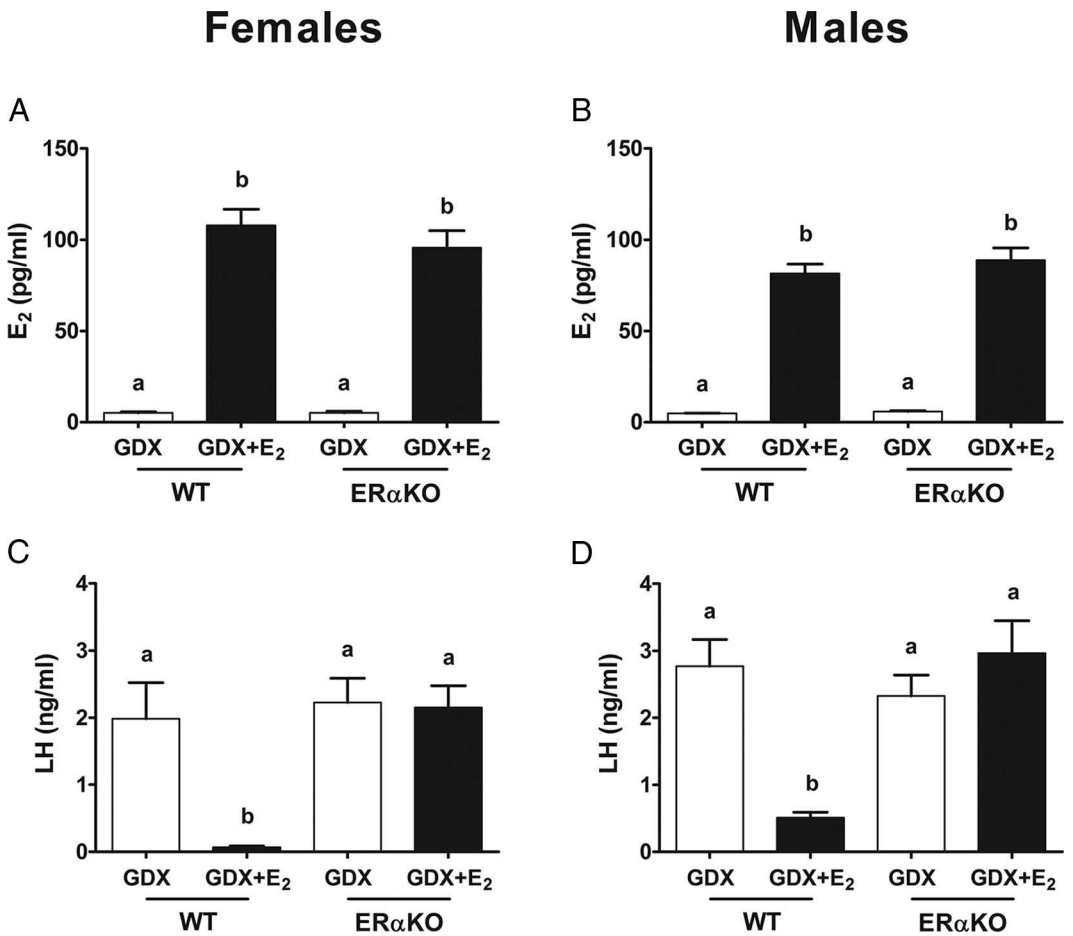

Figure 6. Serum E_2 and LH levels for female and male ERαKO mice. E_2 levels were elevated in both female (A) and male (B) ERαKO and WT mice treated with E_2. However, LH was suppressed by E_2 in female (C) and male (D) WT littermate control, but not in ERαKO females (C) and males (D). Different letters denote significant group differences within each sex ($P < .05$).

lates MeA *Kiss1* and how this compares with the regulation of hypothalamic *Kiss1* populations exclusively by ERα could provide an important framework for future investigations of potential functions of MeA *Kiss1* neurons. Based on this rationale, we used ER knockout mice to examine whether ERα and/or ERβ were necessary for the E_2-induced *Kiss1* expression in the MeA. Previous research in female mice has shown that ERβ is not required for hypothalamic *Kiss1* expression (10), and our present data in both females and males support this, with ERβKO females and males each showing normal E_2-regulated AVPV and ARC *Kiss1* expression patterns comparable with WT mice. Similarly, we found that in the MeA, despite very high ERβ expression in this region, ERβKO mice of both sexes showed a marked increase in *Kiss1* levels with E_2 treatment, comparable with the increase observed in WT mice, indicating that ERβ is also not necessary for E_2-induced MeA *Kiss1* expression.

In contrast to ERβ, ERα is known to be required for the E_2 regulation of AVPV and ARC *Kiss1* (8, 10). Our present data in both male and female ERαKO mice support this previous conclusion. Indeed, in both sexes, we found that despite comparable E_2 levels between E_2-treated WT and ERαKO mice, E_2-treated ERαKOs fail to show any significant increase in *Kiss1* in the AVPV or suppression of *Kiss1* in the ARC. ERα also appears to be required for normal E_2 induction of *Kiss1* in the MeA. We found that in the MeA, as in the hypothalamus, E_2-treated ERαKO mice had significantly fewer *Kiss1* cells than E_2-treated WTs, indicating that ERα is required for the maximal expression of *Kiss1* in the MeA. However, it was interesting to note that E_2-treated ERαKOs had significantly more MeA *Kiss1* neurons than GDX ERαKOs, indicative of a small degree of E_2 up-regulation, perhaps by some other ER, possibly ERβ or G protein-coupled receptor (GPR)-30, that may compensate in the absence of ERα or be sufficient to induce MeA *Kiss1*. This minor up-regulation with E_2 treatment was limited to the MeA *Kiss1* population and not observed in either the AVPV or ARC *Kiss1* populations. ERβ is highly expressed in the MeA, and thus, it is possible that ERβ is sufficient, but not required, for E_2-induced MeA *Kiss1* expression. At this time, there

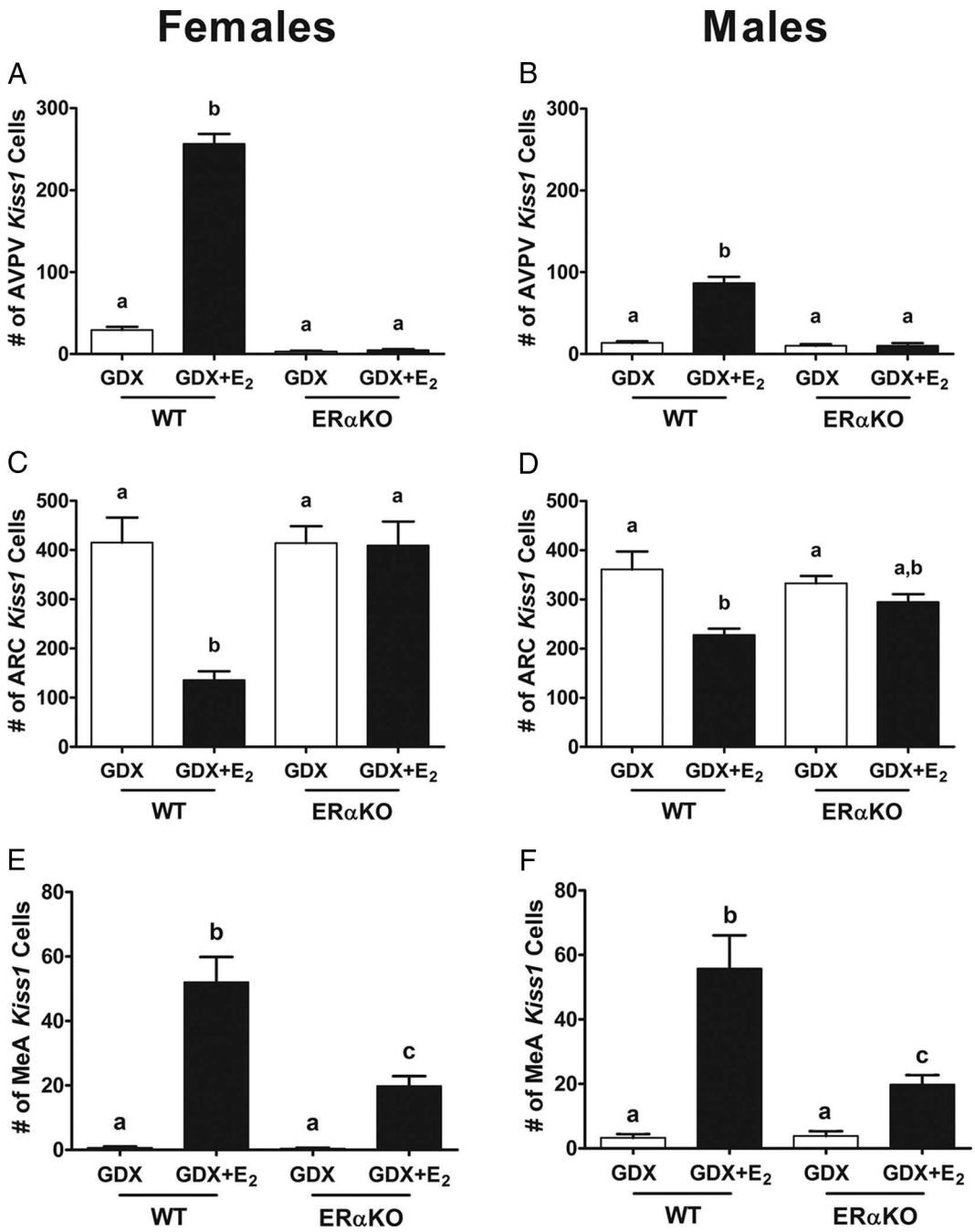

Figure 7. *Kiss1* mRNA expression in ERαKO mice. Mean number of *Kiss1* cells in the AVPV of female (A) and male (B), ARC of female (C) and male (D), and MeA of female (E) and male (F) ERαKO and WT littermates. Different letters denote significant group differences within each sex ($P < .05$).

is not a consistently suitable and well-established positive control for ERβ action in the brain, making it difficult to assess this possibility at present. However, the role of ERβ in this minimal up-regulation of MeA *Kiss1* could be examined in future research by examining E_2-up-regulation in double-ERα/ERβ KO mice. It is also possible that MeA *Kiss1* in WT mice is normally regulated solely via ERα but that the developmental compensation by another ER is induced in the MeA of ERαKO mice, allowing for their minor E_2-stimulated increase in MeA *Kiss1* expression. Lastly, the current experiments indicate that ERα, but not ERβ, is required for MeA *Kiss1* expression, but future research is needed to know whether ERα has a direct or indirect effect on *Kiss1* neurons in the MeA.

The primary known functions of hypothalamic kisspeptin are its roles in stimulating reproduction and mediating sex steroid feedback signaling. Although the MeA has many ascribed functions in multiple diverse physio-

logical and behavioral systems, this region has also been implicated in modulating some aspects of reproduction. Lesions of the MeA disrupt ovarian cycles (15–17), and electrical stimulation of the MeA results in increases in LH (14), mimicking an LH surge, suggesting that in adulthood, some factor in the MeA facilitates reproduction. Thus, it is possible that the mechanism by which the MeA influences reproduction is via kisspeptin signaling arising from this region. ERα is the primary ER regulating both hypothalamic *Kiss1* and reproductive sex steroid feedback loops (8, 10), and our data demonstrate that ERα is also necessary for the complete E_2-induced increase in MeA *Kiss1*. Thus, it is possible that *Kiss1* neurons in the MeA modulate reproductive parameters under the influence of ERα, as occurs for *Kiss1* neurons in the AVPV and ARC. However, the small degree of partial modulation of MeA *Kiss1* by another ER other than ERα may indicate an additional estrogen-mediated role(s) of MeA *Kiss1* neurons beyond reproduction. Recent data demonstrating projections from the accessory olfactory bulb to the MeA *Kiss1* neurons (42) suggest that these neurons may relay chemosensory information, which may indicate a role of MeA *Kiss1* neurons in modulating reproductive and/or nonreproductive behaviors or physiology that are modulated by olfactory cues.

In summary, very little is known about the development and regulation of *Kiss1* neurons in the MeA. Our data are the first to demonstrate that MeA *Kiss1* expression increases around puberty, coincident with developmental increases in sex steroids. We also provide evidence to suggest that the developmental onset and increase in MeA *Kiss1* are likely driven primarily by exposure to rising circulating sex steroids, although we cannot currently rule out that MeA *Kiss1* neurons also undergo additional yet-to-be identified changes during pubertal development that prevent earlier expression. Finally, our data indicate that *Kiss1* in the MeA is regulated primarily by ERα, and not ERβ, and therefore, these neurons are likely involved in mediating ERα-regulated behaviors or physiology, beginning around or after puberty.

Acknowledgments

Address all correspondence and requests for reprints to: Alexander S. Kauffman, PhD, Department of Reproductive Medicine, Leichtag Building, Room 3A-15, University of California, San Diego, 9500 Gilman Drive, #0674, La Jolla, CA 92093. E-mail: akauffman@ucsd.edu.

This work was supported by National Science Foundation Grant IOS-1457226 and *Eunice Kennedy Shriver* National Institute of Child Health and Human Development Specialized Cooperative Centers Program in Reproduction and Infertility Research Grants U54 HD012303 (to the University of California, San Diego) and U54 HD28934 (to the University of Virginia). S.B.Z.S. is supported by Grants F32 HD088060 and T32 HD007203.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Seminara SB, Messager S, Chatzidaki EE, et al.** The GPR54 gene as a regulator of puberty. *N Engl J Med*. 2003;349:1614–1627.
2. **Topaloglu AK, Tello JA, Kotan LD, et al.** Inactivating KISS1 mutation and hypogonadotropic hypogonadism. *N Engl J Med*. 2012; 366:629–635.
3. **Lapatto R, Pallais JC, Zhang D, et al.** Kiss1−/− mice exhibit more variable hypogonadism than Gpr54−/− mice. *Endocrinology*. 2007;148:4927–4936.
4. **de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E.** Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc Natl Acad Sci USA*. 2003;100:10972–10976.
5. **Shahab M, Mastronardi C, Seminara SB, Crowley WF, Ojeda SR, Plant TM.** Increased hypothalamic GPR54 signaling: a potential mechanism for initiation of puberty in primates. *Proc Natl Acad Sci USA*. 2005;102:2129–2134.
6. **Gottsch ML, Cunningham MJ, Smith JT, et al.** A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. *Endocrinology*. 2004;145:4073–4077.
7. **Franceschini I, Lomet D, Cateau M, Delsol G, Tillet Y, Caraty A.** Kisspeptin immunoreactive cells of the ovine preoptic area and arcuate nucleus co-express estrogen receptor α. *Neurosci Lett*. 2006; 401:225–230.
8. **Smith JT, Dungan HM, Stoll EA, et al.** Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse. *Endocrinology*. 2005;146:2976–2984.
9. **Kauffman AS, Gottsch ML, Roa J, et al.** Sexual differentiation of Kiss1 gene expression in the brain of the rat. *Endocrinology*. 2007; 148:1774–1783.
10. **Smith JT, Cunningham MJ, Rissman EF, Clifton DK, Steiner RA.** Regulation of Kiss1 gene expression in the brain of the female mouse. *Endocrinology*. 2005;146:3686–3692.
11. **Kim J, Semaan SJ, Clifton DK, Steiner RA, Dhamija S, Kauffman AS.** Regulation of Kiss1 expression by sex steroids in the amygdala of the rat and mouse. *Endocrinology*. 2011;152:2020–2030.
12. **Kim W, Jessen HM, Auger AP, Terasawa E.** Postmenopausal increase in KiSS-1, GPR54, and luteinizing hormone releasing hormone (LHRH-1) mRNA in the basal hypothalamus of female rhesus monkeys. *Peptides*. 2009;30:103–110.
13. **Xu Z, Kaga S, Mochiduki A, et al.** Immunocytochemical localization of kisspeptin neurons in the rat forebrain with special reference to sexual dimorphism and interaction with GnRH neurons. *Endocr J*. 2012;59:161–171.
14. **Beltramino C, Taleisnik S.** Facilitatory and inhibitory effects of electrochemical stimulation of the amygdala on the release of luteinizing hormone. *Brain Res*. 1978;144:95–107.
15. **Chateau D, Kauffmann MT, Aron C.** Are the amygdaloid projections to the hypothalamic ventromedial nucleus involved in estrous rhythm regulation in the female rat? *Exp Clin Endocrinol*. 1984; 83:303–309.
16. **Velasco ME, Taleisnik S.** Effects of the interruption of amygdaloid and hippocampal afferents to the medial hypothalamus on gonadotrophin release. *J Endocrinol*. 1971;51:41–55.
17. **Tyler JL, Gorski RA.** Effects of corticomedial amydgala lesions or

olfactory bulbectomy on LH responses to ovarian steroids in the female rat. *Biol Reprod*. 1980;22:927–934.
18. **DiBenedictis BT, Ingraham KL, Baum MJ, Cherry JA.** Disruption of urinary odor preference and lordosis behavior in female mice given lesions of the medial amygdala. *Physiol Behav*. 2012;105:554–559.
19. **Maras PM, Petrulis A.** Chemosensory and steroid-responsive regions of the medial amygdala regulate distinct aspects of opposite-sex odor preference in male Syrian hamsters. *Eur J Neurosci*. 2006; 24:3541–3552.
20. **Kauffman AS, Navarro VM, Kim J, Clifton DK, Steiner RA.** Sex differences in the regulation of Kiss1/NKB neurons in juvenile mice: implications for the timing of puberty. *Am J Physiol Endocrinol Metab*. 2009;297:E1212–E1221.
21. **Roa J, Vigo E, Castellano JM, et al.** Opposite roles of estrogen receptor (ER)-α and ERβ in the modulation of luteinizing hormone responses to kisspeptin in the female rat: implications for the generation of the preovulatory surge. *Endocrinology*. 2008;149:1627–1637.
22. **Shughrue PJ, Scrimo PJ, Merchenthaler I.** Evidence for the colocalization of estrogen receptor-β mRNA and estrogen receptor-α immunoreactivity in neurons of the rat forebrain. *Endocrinology*. 1998;139:5267–5270.
23. **Osterlund M, Kuiper GG, Gustafsson JA, Hurd YL.** Differential distribution and regulation of estrogen receptor-α and -β mRNA within the female rat brain. *Brain Res Mol Brain Res*. 1998;54:175–180.
24. **Di Giorgio NP, Semaan SJ, Kim J, et al.** Impaired GABAB receptor signaling dramatically up-regulates Kiss1 expression selectively in nonhypothalamic brain regions of adult but not prepubertal mice. *Endocrinology*. 2014;155:1033–1044.
25. **Cao J, Patisaul HB.** Sex-specific expression of estrogen receptors α and β and Kiss1 in the postnatal rat amygdala. *J Comp Neurol*. 2013;521:465–478.
26. **Semaan SJ, Kauffman AS.** Daily successive changes in reproductive gene expression and neuronal activation in the brains of pubertal female mice. *Mol Cell Endocrinol*. 2015;401:84–97.
27. **Clarkson J, Herbison AE.** Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons. *Endocrinology*. 2006; 147:5817–5825.
28. **Desroziers E, Droguerre M, Bentsen AH, et al.** Embryonic development of kisspeptin neurones in rat. *J Neuroendocrinol*. 2012;24: 1284–1295.
29. **Kumar D, Periasamy V, Freese M, Voigt A, Boehm U.** In utero development of kisspeptin/GnRH neural circuitry in male mice. *Endocrinology*. 2015;156:3084–3090.
30. **Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P, Mark M.** Effect of single and compound knockouts of estrogen receptors α (ERα) and β (ERβ) on mouse reproductive phenotypes. *Development*. 2000;127:4277–4291.
31. **Stephens SB, Tolson KP, Rouse ML Jr, et al.** Absent progesterone signaling in kisspeptin neurons disrupts the LH surge and impairs fertility in female mice. *Endocrinology*. 2015;156:3091–3097.
32. **Kim J, Tolson KP, Dhamija S, Kauffman AS.** Developmental GnRH signaling is not required for sexual differentiation of kisspeptin neurons but is needed for maximal Kiss1 gene expression in adult females. *Endocrinology*. 2013;154:3273–3283.
33. **Russo KA, LA JL, Stephens SB, et al.** Circadian control of the female reproductive axis through gated responsiveness of the RFRP-3 system to VIP signaling. *Endocrinology*. 2015;156:2608–2618.
34. **Luo E, Stephens SB, Chaing S, Munaganuru N, Kauffman AS, Breen KM.** Corticosterone blocks ovarian cyclicity and the LH surge via decreased kisspeptin neuron activation in female mice. *Endocrinology*. 2016;157:1187–1199.
35. **Dror T, Franks J, Kauffman AS.** Analysis of multiple positive feedback paradigms demonstrates a complete absence of LH surges and GnRH activation in mice lacking kisspeptin signaling. *Biol Reprod*. 2013;88:146.
36. **Shughrue PJ, Lane MV, Merchenthaler I.** Comparative distribution of estrogen receptor-α and -β mRNA in the rat central nervous system. *J Comp Neurol*. 1997;388:507–525.
37. **Dhillo WS, Chaudhri OB, Patterson M, et al.** Kisspeptin-54 stimulates the hypothalamic-pituitary gonadal axis in human males. *J Clin Endocrinol Metab*. 2005;90:6609–6615.
38. **Navarro VM, Castellano JM, Fernandez-Fernandez R, et al.** Characterization of the potent luteinizing hormone-releasing activity of KiSS-1 peptide, the natural ligand of GPR54. *Endocrinology*. 2005; 146:156–163.
39. **Navarro VM, Castellano JM, Fernandez-Fernandez R, et al.** Effects of KiSS-1 peptide, the natural ligand of GPR54, on follicle-stimulating hormone secretion in the rat. *Endocrinology*. 2005;146: 1689–1697.
40. **Irwig MS, Fraley GS, Smith JT, et al.** Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat. *Neuroendocrinology*. 2004;80:264–272.
41. **Messager S, Chatzidaki EE, Ma D, et al.** Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. *Proc Natl Acad Sci USA*. 2005;102:1761–1766.
42. **Pineda R, Plaisier F, Millar RP, Ludwig M.** Amygdala kisspeptin neurons: putative mediators of olfactory control of the gonadotropic axis. *Neuroendocrinology*. In press.