Estradiol-Dependent and -Independent Stimulation of *Kiss1* Expression in the Amygdala, BNST, and Lateral Septum of Mice

Shannon B.Z. Stephens,[1]* Noelia P. Di Giorgio,[2]* Reanna B. Liaw,[1] Ruby A. Parra,[1] Jennifer A. Yang,[1] Navdeep Chahal,[1] Victoria A. Lux-Lantos,[2] and Alexander S. Kauffman[1]

[1]Department of Obstetrics, Gynecology, and Reproductive Sciences, University of California, San Diego, La Jolla, California 92093; and [2]Institute of Biology and Experimental Medicine, National Scientific and Technical Research Council, CP 1428 Buenos Aires, Argentina

Kisspeptin, encoded by *Kiss1*, activates reproduction by stimulating GnRH neurons. Although most *Kiss1* neurons are located in the hypothalamus, smaller *Kiss1* populations also reside in the medial amygdala (MeA), bed nucleus of the stria terminalis (BnST), and lateral septum (LS). However, very little is known about the regulation and function of these extra-hypothalamic *Kiss1* neurons. This study focused on the roles and interactions of two signaling factors, estradiol (E_2) and GABA, known to stimulate and inhibit, respectively, extra-hypothalamic *Kiss1* expression. First, using estrogen receptor (ER)α knockout (KO) and βERKO mice, we demonstrated that *Kiss1* in both the BnST and LS is stimulated by E_2, as occurs in the MeA, and that this E_2 upregulation occurs via ERα, but not ERβ. Second, using $GABA_BR$ KO and wild-type mice, we determined that whereas E_2 normally increases extra-hypothalamic *Kiss1* levels, such upregulation by E_2 is further enhanced by the concurrent absence of $GABA_BR$ signaling in the MeA and LS, but not the BnST. Third, we demonstrated that when $GABA_BR$ signaling is absent, the additional removal of gonadal sex steroids does not abolish *Kiss1* expression in the MeA and BnST, and in some cases the LS. Thus, *Kiss1* expression in these extra-hypothalamic regions is not solely dependent on E_2 stimulation. Finally, we demonstrated a significant positive correlation between *Kiss1* levels in the MeA, BnST, and LS, but not between these regions and the hypothalamus (anteroventral periventricular nucleus/periventricular nucleus). Collectively, our findings indicate that both E_2 and GABA independently regulate all three extra-hypothalamic *Kiss1* populations, but their regulatory interactions may vary by brain region and additional yet-to-be-identified factors are likely involved. **(*Endocrinology* 159: 3389–3402, 2018)**

The neuropeptide kisspeptin, encoded by the *Kiss1* gene, and its receptor, Kiss1r, are required for mammalian reproduction. This is supported by findings that mutations in these genes in humans and rodents lead to deficits in puberty and adult fertility (1–5). *Kiss1* neurons are most abundantly located in the hypothalamus, in the anteroventral periventricular (AVPV), periventricular (PeN), and arcuate (ARC) nuclei (5–10), although smaller populations of *Kiss1* neurons are also present in extra-hypothalamic areas such as the medial amygdala (MeA), bed nucleus of the stria terminalis (BnST), and lateral septum (LS) (5, 11–16). Most kisspeptin research to date has focused on the regulation and reproductive functions of hypothalamic *Kiss1* neurons, and there is still very little known about the regulation and function of extra-hypothalamic *Kiss1* neurons. The MeA, BnST, and LS regions have numerous behavioral and physiological functions, including but not limited to effects on reproductive physiology and behavior (17–30). Therefore, understanding the regulation of these

ISSN Online 1945-7170

Received 14 June 2018. Accepted 6 August 2018.
First Published Online 9 August 2018

*These authors contributed equally to this study.

Abbreviations: ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus; BnST, bed nucleus of the stria terminalis; E_2, estradiol; ER, estrogen receptor; GDX, gonadectomized; ISH, *in situ* hybridization; KO, knockout; LS, lateral septum; MeA, medial amygdala; PeN, periventricular nucleus; T, testosterone; WT, wild-type.

extra-hypothalamic *Kiss1* populations may provide valuable insight into their possible functions.

Kiss1 gene expression in the ARC and AVPV/PeN is differentially regulated by gonadal sex steroids [testosterone (T) and estradiol (E_2)] (8–10). In the ARC, *Kiss1* expression increases following the removal of gonadal sex steroids via gonadectomy and decreases following exogenous T or E_2 treatment, supporting the hypothesis that ARC *Kiss1* neurons participate in sex steroid negative feedback control of GnRH pulses (8–10). In contrast to the ARC, in the AVPV/PeN, *Kiss1* expression is reduced with gonadectomy and increased following E_2 treatment, supporting a role for AVPV/PeN *Kiss1* neurons in participating in E_2-mediated positive feedback control of ovulation in females (8–10). Similar to AVPV/PeN *Kiss1* expression, MeA *Kiss1* expression is dramatically reduced following gonadectomy, whereas E_2 or T treatment robustly increases MeA *Kiss1* expression (11, 31). DHT treatment has no effect on MeA *Kiss1* levels, indicating that *Kiss1* expression in the MeA is upregulated by sex steroids specifically via estrogen-dependent pathways (11). In both the hypothalamus and the MeA, this E_2 regulation of *Kiss1* expression occurs primarily via estrogen receptor (ER)α (9, 10, 31, 32). However, it is currently unknown whether the BnST and LS *Kiss1* populations are also regulated by E_2, whether any potential E_2 regulation in these regions is stimulatory or inhibitory, and whether such E_2 regulation occurs via ERα or another ER, such as ERβ. The LS only expresses ERα, suggesting that any direct E_2 regulation of *Kiss1* expression would likely occur via ERα (33). However, the BnST expresses both ERα and ERβ, indicating either (or both) ER may possibly regulate BnST *Kiss1* expression (33–36), but this remains undetermined.

In addition to regulation by E_2, we previously demonstrated that *Kiss1* levels in the MeA, BnST, and LS are also regulated by GABA signaling. Specifically, removal of $GABA_BR$ signaling via global $GABA_BR$ knockout (KO) greatly increases *Kiss1* expression in the MeA, BnST, and LS in gonad-intact mice of both sexes (15). This suggests that endogenous GABA acting through $GABBA_BR$ normally acts to reduce *Kiss1* expression in the MeA, BnST, and LS. Interestingly, AVPV/PeN and ARC *Kiss1* levels did not differ between wild-type (WT) and $GABA_BR$ KO mice, indicating that $GABA_BR$ regulation of *Kiss1* expression is limited to extra-hypothalamic populations such as the MeA, BnST, and LS. However, it has not been determined whether such GABA regulation of extra-hypothalamic *Kiss1* is dependent on E_2. Specifically, it is unknown whether E_2, which increases MeA *Kiss1* levels in WT mice, further increases extra-hypothalamic *Kiss1* expression in $GABA_BR$ KO mice or whether their *Kiss1* expression is already maximized by the absence of GABA signaling. Second, it is unknown whether *Kiss1* levels in the MeA, BnST, and LS of $GABA_BR$ KOs are undetectable when sex steroids are absent, as is the normally case for WT mice, or whether reduced $GABA_BR$ signaling is able to stimulate *Kiss1* expression even without E_2 present.

This study had three main goals to address how E_2 and $GABA_BR$ signaling regulate extra-hypothalamic *Kiss1*

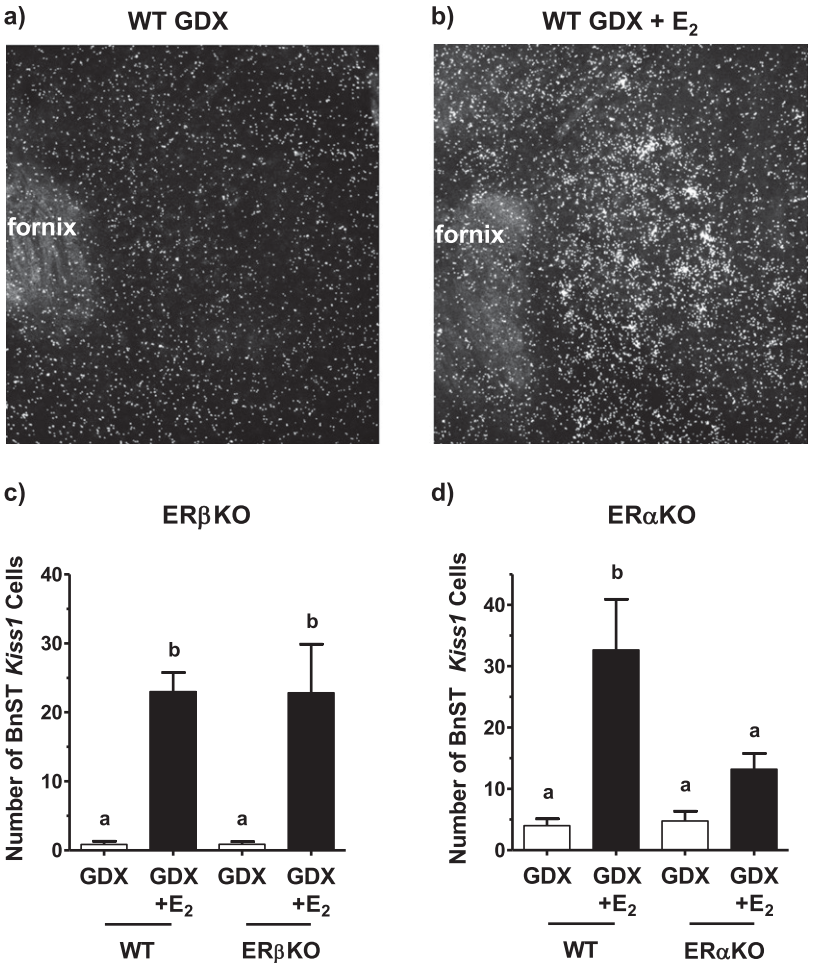

Figure 1. *Kiss1* mRNA expression in the BnST of βERKO and ERαKO mice. Representative images of (a) essentially absent BnST *Kiss1* expression in a GDX WT male and (b) elevated BnST *Kiss1* expression in a WT E_2-treated male. (c) Both WT and βERKO male mice showed a significant increase in the number of BnST *Kiss1* cells with E_2 treatment, but (d) ERαKO male mice failed to show an increase in BnST *Kiss1* expression with E_2 treatment (n = 6 to 9 per group). Different letters denote significant group differences ($P < 0.05$).

expression. First, we examined whether *Kiss1* expression in the BnST and LS is regulated by E_2, whether such regulation is stimulatory (as is the case for the MeA and AVPV/PeN) or inhibitory (as is the case for the ARC), and whether any such E_2 regulation occurs via ERα or ERβ pathways. Second, we examined the effects of exogenous E_2 treatment on *Kiss1* expression in the MeA, BnST, and LS of $GABA_BR$ KOs and WTs to determine whether E_2 exposure further increases the already elevated *Kiss1* expression of $GABA_BR$ KO mice beyond that of similarly treated WT mice. Third, we examined *Kiss1* expression in the MeA, BnST, and LS of gonadectomized (GDX) $GABA_BR$ KO and WT mice to determine whether the absence of sex steroids would fully suppress *Kiss1* expression in $GABA_BR$ KO mice, as it does in WT mice, or whether *Kiss1* expression remains elevated in the absence of $GABA_BR$ signaling despite the lack of E_2.

Materials and Methods

Animals

Experiments used either male ERαKO, βERKO, and WT littermates (C57BL/6 background; experiment 1) or $GABA_{B1}R$ KO mice and WT littermates of both sexes (BALB/c background; experiments 2 and 3). As in our previous studies, $GABA_{B1}R$ heterozygous mice were bred to produce $GABA_{B1}R$ KO ($GABA_BR$ KO) mice, which globally lack a functional $GABA_{B1}$ receptor, and WT littermates (15, 37–41). Heterozygous ERαKO mice (originally created by the Chambon laboratory) (42) were bred to produce mice lacking ERα (ERαKO) and WT littermates. Mice lacking ERβ (βERKO) and WT littermates were produced by breeding heterozygous βERKO mice. Mice were housed two to three mice per cage in a 12-hour light: 12-hour dark cycle, with *ad libitum* access to food and water. All experiments were performed on adult mice. Surgeries were performed on mice that were anesthetized with isoflurane or ketamine. All experimental procedures were approved by the local Institutional Animal Care and Use Committees (Institute of Biology and Experimental Medicine, National Scientific and Technical Research Council for $GABA_{B1}R$ KO mice; University of California, San Diego for ERαKO and βERKO mice).

Surgeries, hormone treatment, and tissue collection

For all experiments, all mice of both sexes were bilaterally GDX when under isoflurane or ketamine/xylazine cocktail anesthesia. In some cases (see specific experiments), GDX mice were also surgically implanted subcutaneously with a Silastic capsule (8 mm length; inner diameter, 1.47 mm; outer diameter, 1.96 mm) containing E_2 (2 mm of a 1:25 mixture of E_2 to cholesterol) or no hormonal treatment (control) at the time of gonadectomy. This E_2 dosage has previously been shown to produce elevated circulating E_2 levels, properly inhibit LH secretion (negative feedback), and successfully alter *Kiss1* gene expression in mice (8, 11, 31).

At the end of each experiment, mice were lightly anesthetized and blood samples collected. The mice were then rapidly decapitated for brain collection. Ninety minutes after blood collection, samples were centrifuged (15 minutes at 5000 rpm) and the serum was collected and stored at −20°C. Blood serum samples for ERαKO and βERKO mice (experiment 1) were assayed in singlet for LH via a sensitive mouse LH RIA (lower detection limit, 0.04 ng/mL; average reportable range, 0.04 to 75 ng/mL) at the University of Virginia's Ligand Assay and Analysis Core. Blood serum samples of $GABA_BR$ KO and WT mice (experiments 2 and 3) were assayed for LH in duplicates by RIA with a kit from National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, and Dr. A.F. Parlow (assay detection limits, 0.2 ng/mL to 107 ng/mL; intra-assay and interassay coefficients of variation, LH 7.2% and 11.4%). Brains were collected immediately following decapitation, frozen on dry ice, and then stored at −80°C. Brains were cut on a cryostat into 20-μm coronal sections, spanning the entire anterior hypothalamus/forebrain through the caudal end of the hypothalamus and amygdala, across five sets of slides and mounted onto SuperFrost Plus slides. Slides were stored at −80°C until *in situ* hybridization (ISH).

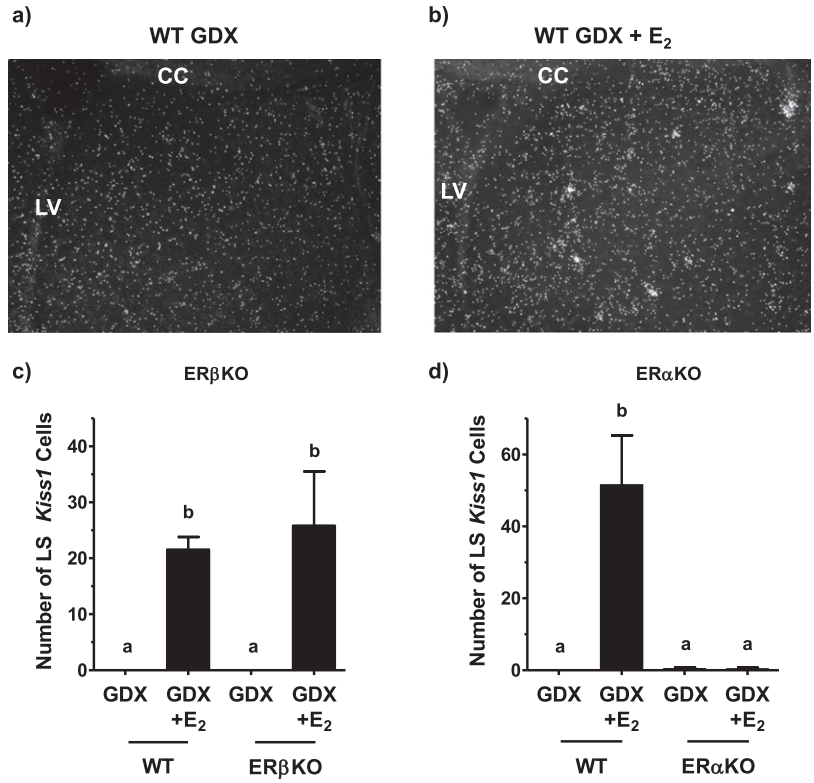

Figure 2. *Kiss1* mRNA expression in the LS of βERKO and ERαKO mice. Representative images of (a) absent LS *Kiss1* expression in a GDX WT male and (b) notable LS *Kiss1* expression in a WT E_2-treated male. E_2 treatment (c) increased LS *Kiss1* expression in both WT and βERKO mice, but (d) failed to increase LS *Kiss1* expression in ERαKO mice (n = 6 to 9 mice per group). Different letters denote significant group differences ($P < 0.05$). CC, corpus callosum; LV, lateral ventricle.

Single-label ISH

Single-label ISH assays for *Kiss1* expression were performed on one set of slides using a well-established radiolabeled (33P) *Kiss1* riboprobe, as described previously (11, 31, 43, 44). Briefly, slides were fixed in 4% paraformaldehyde, treated with acetic anhydride, rinsed in 2× SSC, and dehydrated in ethanol washes. Slides were then washed in chloroform, dehydrated in additional ethanol washes, and then air dried for 90 minutes before the application of the *Kiss1* riboprobe. Radiolabeled (33P) *Kiss1* (0.04 pmol/mL) antisense riboprobe was added to tRNA, heat denatured, and then added to hybridization buffer. This probe mixture was then applied to each slide (100 μL per slide) prior to overnight hybridization in a 55°C humidity chamber. The following day, slides were washed in 4× SSC at room temperature, treated with RNase A for 30 minutes at 37°C, and then washed in an RNase buffer for 30 minutes at 37°C. Slides were then washed for 30 minutes at room temperature in 2× SSC, washed for 1 hour in 0.1× SSC at 62°C, and dehydrated in ethanol washes. Following 90 minutes of air drying, the slides were dipped in Kodak NTB emulsion, air dried for another 90 minutes, and then stored at 4°C until developing 7 to 11 days later, depending on the experiment. Owing to the large size of the assays, ERαKO and βERKO brains for experiment 1 and male and female brains for experiments 2 and 3 were run in separate ISH assays.

For the ISH assays, *Kiss1* expression levels in each brain region (AVPV/PeN, ARC, MeA, BnST, and LS) were measured using computer-assisted microscopy using a well-established counting software (Grains; Dr. Don Clifton, University of Washington) (9–11, 31, 44–46). For this, an automated silver grains imaging processing system connected to a dark field microscope counts the number of *Kiss1* cells, defined as discrete clusters of P33-induced silver grains at least threefold greater than background. To ensure unbiased measurements, microscopy and counting analyses were completed by an investigator blinded to genotype and hormonal treatment. For each of the brain regions, *Kiss1* expression in the entire bilateral region was counted, and the ISH images shown are representative images of *Kiss1* expression in each brain region.

Experiment 1: Does E_2 alter *Kiss1* expression in the LS and BnST and, if so, does this occur via ERα or ERβ?

Although *Kiss1* expression has been noted in the LS and BnST of gonad-intact rodents (5, 13–16), it is unknown (1) whether these two *Kiss1* populations are regulated by E_2, as are the hypothalamic and MeA *Kiss1* populations; (2) whether any E_2 regulation of BnST and LS *Kiss1* is stimulatory (as in the MeA and AVPV/PeN) or inhibitory (as in the ARC); and (3) whether such E_2 regulation of these two *Kiss1* populations occurs via ERα and/or ERβ. Using ERαKO and βERKO male mice, as well as their WT littermates, this experiment determined whether E_2 treatment upregulates or reduces LS and BnST *Kiss1* expression, and whether either ERα or ERβ are necessary for this regulation. Adult (7 weeks) male ERαKO, βERKO, and their respective WT littermates were GDX and 1 week later received either a Silastic E_2 capsule or no hormonal treatment (GDX controls) (n = 6 to 9 per group). After 5 days of E_2 exposure, all mice were killed and blood and brains were collected to measure circulating LH levels (to ensure proper E_2 implant effectiveness) and *Kiss1* expression in the brain (BnST and LS), respectively.

Experiment 2: Does E_2 treatment increase extra-hypothalamic *Kiss1* expression to the same levels in $GABA_BR$ KO and WT mice?

Kiss1 expression in the MeA is strongly upregulated by E_2 (11, 31), and experiment 1 above determined that this E_2 upregulation also occurs in the BnST and LS. Besides E_2 regulation, diminished $GABA_BR$ signaling also dramatically increases *Kiss1* expression in the MeA, BnST, and LS in gonad-intact mice (15). However, it is unknown whether the stimulatory effects of absent $GABA_BR$ can be further amplified with E_2 exposure, which strongly increases MeA, BnST, and LS *Kiss1* levels in WT mice. This experiment tested: (1) whether E_2 treatment increases MeA *Kiss1* expression in $GABA_BR$ KO mice

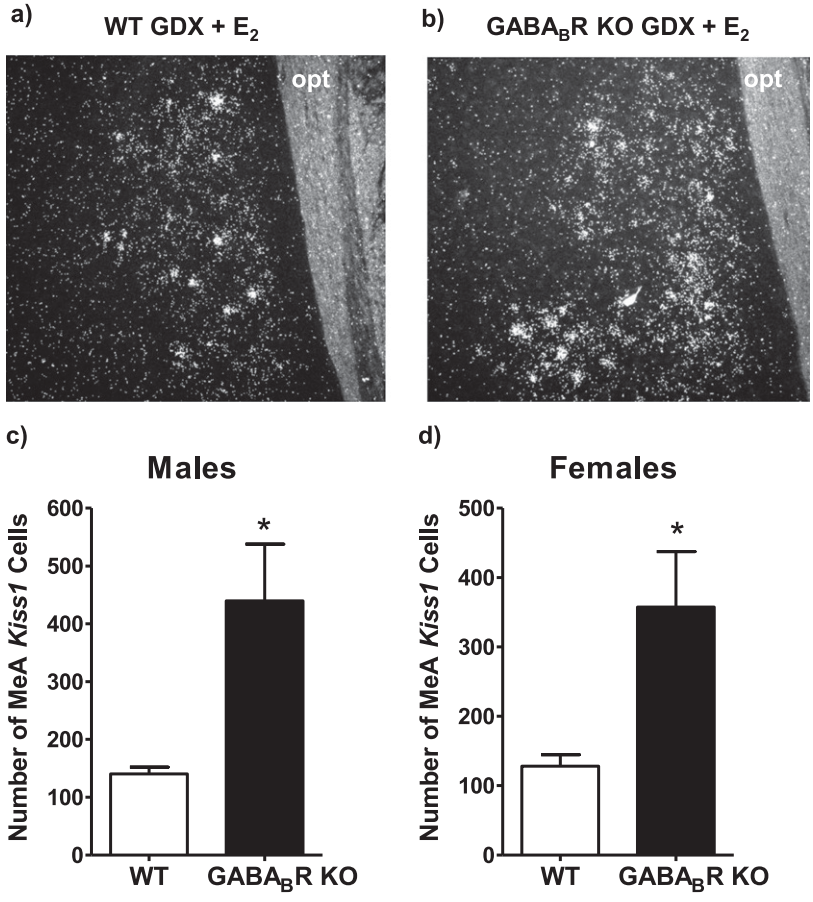

Figure 3. *Kiss1* mRNA expression in the MeA of E_2-treated WT and $GABA_BR$ KO mice. Representative MeA *Kiss1* expression in a (a) WT and (b) $GABA_BR$ KO male mouse. E_2-treated $GABA_BR$ KO (c) males and (d) females had more *Kiss1* cells in the MeA than did E_2-treated WT mice (n = 7 to 10 mice per group). $^*P < 0.05$. opt, optic tract.

in comparison with GDX $GABA_BR$ KO mice lacking E_2; and (2) whether simultaneous E_2 treatment and absent $GABA_BR$ signaling have additive effects, resulting in even higher *Kiss1* expression in the MeA, BnST, and LS in comparison with E_2-treated WT mice. Adult $GABA_BR$ KO mice and WT littermates were GDX and given a Silastic capsule containing E_2, as in experiment 1. Mice received E_2 treatment 1 week before blood and brains were collected to measure blood LH levels and neural *Kiss1* expression in both extra-hypothalamic (MeA, BnST, LS) and hypothalamic (AVPV/PeN, ARC) brain regions (n = 6 to 11 per group). In a separate assay, we compared *Kiss1* expression in the MeA of GDX and GDX plus E_2 $GABA_BR$ KO male mice (n = 5 to 6 per group).

Experiment 3: Does diminished $GABA_BR$ signaling stimulate extra-hypothalamic *Kiss1* expression even in the absence of gonadal sex steroids?

Kiss1 expression in the MeA (11, 31) and the BnST and LS (experiment 1) is virtually absent in GDX WT mice. However, *Kiss1* expression in these three regions is strongly increased by diminished GABA signaling via $GABA_BR$, as gonad-intact $GABA_BR$ KO mice have greater *Kiss1* expression in these areas than do gonad-intact WT mice (15). It is not known whether this upregulation of *Kiss1* in $GABA_BR$ KO mice is dependent on the presence of gonadal sex steroids, because *Kiss1* levels are normally undetectable in GDX WT mice. In experiment 3, adult $GABA_BR$ KO mice and WT littermates (n = 6 to 9 per group) were GDX and remained untreated (no E_2 exposure) for 1 week. Blood and brains were then collected to examine LH levels (confirming absence of sex steroid feedback) and *Kiss1* expression in the MeA, BnST, LS, AVPV/PeN, and ARC.

Statistical analysis

Data are expressed as the mean ± SEM. A two-way ANOVA and Bonferroni *post hoc* tests were used to compare *Kiss1* expression in the LS and BnST of ERαKO and βERKO mice and their WT littermates (experiment 1). Mann–Whitney *U* tests were used to compare *Kiss1* expression levels of WT and $GABA_BR$ KO mice under the same hormonal milieu for the MeA, BnST, and LS (experiment 2 only). There was no *Kiss1* expression in the LS of WT GDX mice, and thus a Wilcoxon signed rank test was used to compare LS *Kiss1* expression in $GABA_BR$ KO mice to a theoretical median of 0. Unpaired *t* tests were used to examine AVPV/PeN *Kiss1* expression between WT and $GABA_BR$ KO mice under the same hormonal status. Regression analysis was used to examine potential relationships between MeA *Kiss1* expression and *Kiss1* expression in the other brain areas (AVPV/PeN, BnST, and LS). Statistical significance was set at $P < 0.05$.

Results

Experiment 1: E_2 upregulates *Kiss1* expression in the BnST and LS and this occurs via ERα

This experiment examined: (1) whether *Kiss1* cells in the BnST and LS are stimulated by E_2, such as MeA and AVPV/PeN *Kiss1* cells, or inhibited by E_2, such as ARC *Kiss1* cells; and (2) whether ERα and/or ERβ are required for any E_2 regulation of BnST or LS *Kiss1* expression. Functionality of the E_2 implants was verified by assessing serum LH and has previously been reported for these mice: as expected, circulating LH was low in all GDX mice with E_2 implants (signifying proper E_2 negative feedback) and LH levels did not differ between genotypes (31). In the brain, we found that E_2 treatment stimulated *Kiss1* expression in both the BnST and LS. Specifically, in

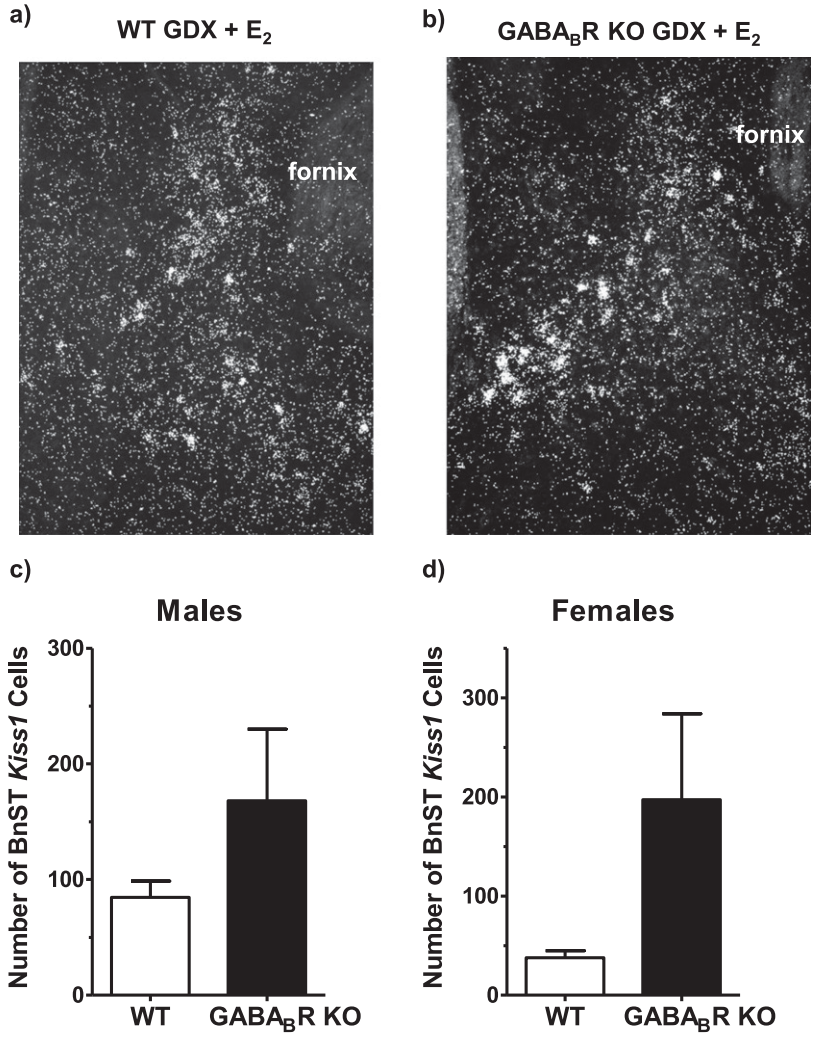

Figure 4. *Kiss1* mRNA expression in the BnST of E_2-treated WT and $GABA_BR$ KO mice. Representative images of (a) WT and (b) $GABA_BR$ KO males with BnST *Kiss1* expression. With E_2 treatment, WT and $GABA_BR$ KO (c) males and (d) females expressed a comparable number of BnST *Kiss1* cells (n = 6 to 11 mice per group).

WT male mice, *Kiss1* expression was very low or completely absent in the BnST and LS of GDX mice, whereas E_2 treatment significantly increased *Kiss1* expression in both brain regions ($P < 0.05$; Figs. 1 and 2). Similar to WT mice, GDX βERKO male mice showed very low or absent *Kiss1* expression in the BnST (Fig. 1) and LS (Fig. 2) without E_2 but had significantly increased *Kiss1* expression in each region with E_2 treatment ($P < 0.05$; Figs. 1 and 2), indicating that ERβ is not required for E_2 upregulation of *Kiss1* expression in the BnST and LS. In contrast to WT and βERKO mice, ERαKO male mice had very low or absent *Kiss1* expression in the BnST (Fig. 1) and LS (Fig. 2) regardless of hormonal treatment, indicating that ERα is required for E_2 upregulation of BnST and LS *Kiss1* expression, similar to previous reports for *Kiss1* in the MeA (31).

Experiment 2: MeA and LS *Kiss1* expression are further elevated in $GABA_BR$ KO mice with E_2 treatment

E_2 significantly increases *Kiss1* expression in the MeA (11, 31), as well as the BnST and LS (experiment 1). Likewise, removal of $GABA_BR$ signaling in gonad-intact mice also increases *Kiss1* expression in the MeA, BnST, and LS, with no effect on hypothalamic AVPV/PeN and ARC *Kiss1* expression (15). This experiment tested whether E_2 treatment: (1) increases MeA *Kiss1* expression in $GABA_BR$ KO mice in comparison with GDX $GABA_BR$ KO mice lacking any E_2; and (2) further increases MeA, BnST, and LS *Kiss1* expression in $GABA_BR$ KO mice above that of E_2-treated WT mice (or is *Kiss1* expression already maximal with E_2 treatment, *i.e.*, a ceiling effect?). As expected, LH levels were low in all E_2-treated WT and $GABA_BR$ KO mice and comparable between genotypes (data not shown), indicative of successfully elevated circulating E_2 levels from the E_2 implants. In the brain, we found that E_2 treatment significantly increased MeA *Kiss1* cell number in GDX $GABA_BR$ KO males vs GDX $GABA_BR$ KO males lacking E_2 (GDX, 10.2 ± 4.2; GDX + E_2, 107.8 ± 45.5; $P < 0.05$). Thus, E_2 upregulation of extrahypothalamic *Kiss1* expression also occurs in $GABA_BR$ KO mice, as it does in WT mice. We also found that *Kiss1* expression in the MeA was significantly higher in E_2-treated $GABA_BR$ KO mice vs E_2-treated WT mice ($P < 0.05$; Fig. 3). This was true for $GABA_BR$ KO mice of both sexes. Thus, the elevated MeA *Kiss1* expression by E_2 does not preclude additional further upregulation by diminished $GABA_BR$ signaling. In the BnST, there was a similar pattern observed between E_2-treated WT and $GABA_BR$ KO mice but it was not significantly different in either sex (Fig. 4), although this may be due in part to low statistical power and high variability in the KO mice. In the LS, as in the MeA, *Kiss1* was significantly higher in E_2-treated $GABA_BR$ KO males than in WT males ($P < 0.05$) and nearly significant in females ($P = 0.07$), again indicating that E_2 and $GABA_BR$ signaling may have independent effects on *Kiss1* levels in the LS (Fig. 5). Unlike in the extrahypothalamic regions, *Kiss1* expression in the AVPV/PeN (Fig. 6) and ARC (data not shown) was not

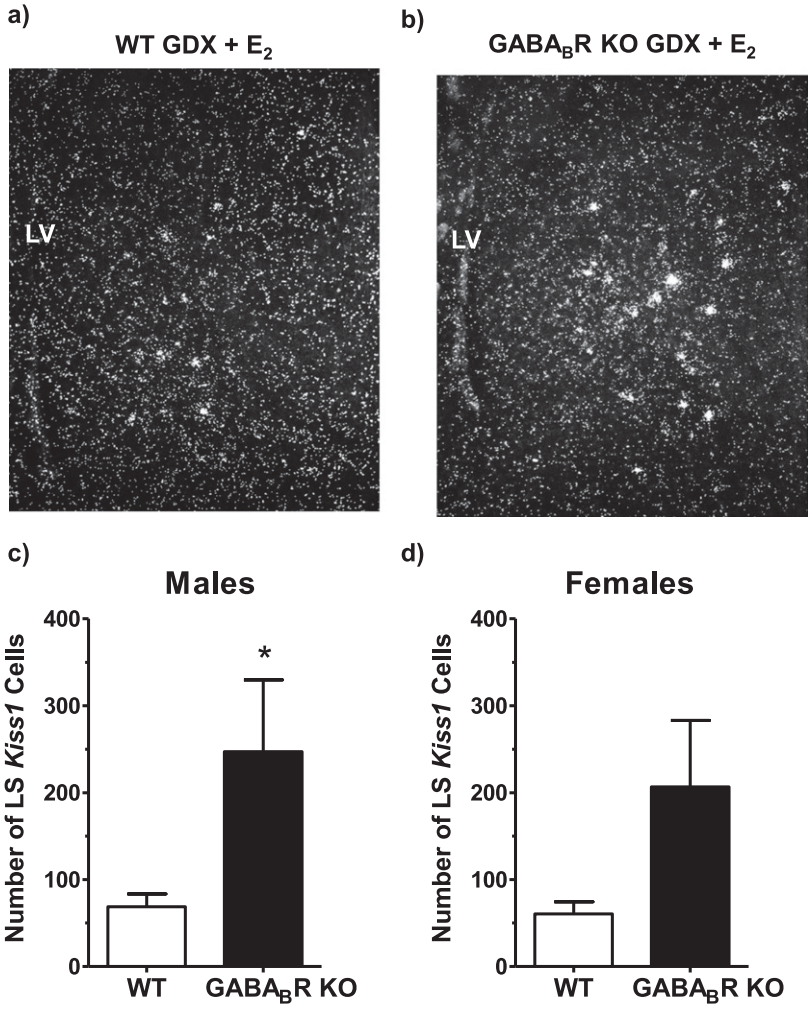

Figure 5. *Kiss1* mRNA expression in the LS of E_2-treated WT and $GABA_BR$ KO mice. Representative LS *Kiss1* expression in a (a) WT and (b) $GABA_BR$ KO male with E_2 treatment. (c) E_2-treated $GABA_BR$ KO males had more LS *Kiss1* cells than did E_2-treated WT males, whereas (d) a similar, but nonsignificant trend ($P < 0.10$), was found between $GABA_BR$ KO females and WT females (n = 7 to 11 mice per group). *$P < 0.05$. LV, lateral ventricle.

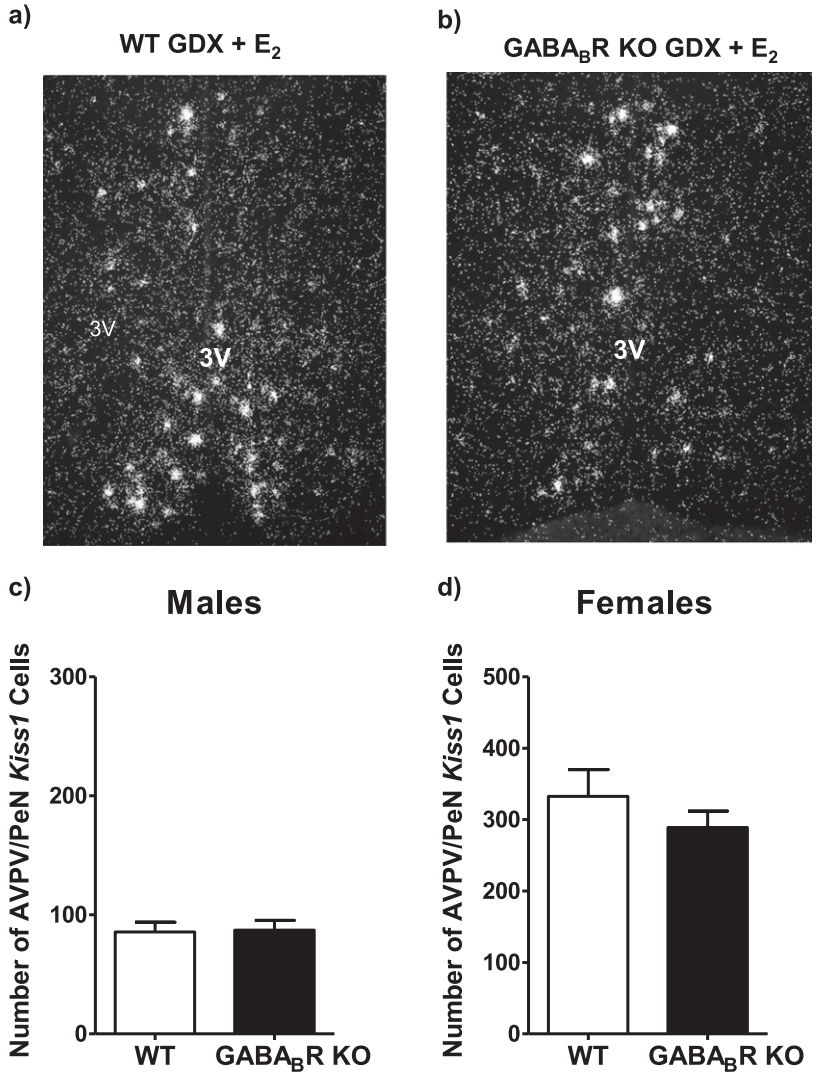

Figure 6. AVPV/PeN *Kiss1* mRNA expression in E_2-treated WT and $GABA_BR$ KO mice. Representative AVPV/PeN *Kiss1* expression in an (a) E_2-treated WT and (b) E_2-treated $GABA_BR$ KO male. AVPV/PeN *Kiss1* expression in (c) males and (d) females did not differ between genotype (n = 6 to 11 mice per group). 3V, third ventricle.

different between E_2-treated $GABA_BR$ KO and WT mice, consistent with our previous findings in gonad-intact mice (15). Thus, unlike the MeA, BnST, and LS, hypothalamic *Kiss1* is not regulated by $GABA_BR$ signaling, regardless of the sex steroid milieu.

During the course of our data analysis, we noticed that even within the same genotype, mice that had very high *Kiss1* expression in the MeA often seemed to also have high BnST and LS expression, whereas mice with lower MeA *Kiss1* expression often had lower BnST and LS expression, despite all mice having a similar E_2 hormonal milieu. We therefore hypothesized that this was a meaningful correlation related to how the extra-hypothalamic *Kiss1* cells are regulated. We hypothesized that the number of *Kiss1* cells in the MeA would be positively related to the number of cells in the BnST and LS, but have no correlation with AVPV/PeN *Kiss1* cells number. Indeed, regression analysis of all data from E_2-treated males of both genotypes determined that there was a significant, positive relationship between MeA *Kiss1* cells and BnST *Kiss1* cells ($P < 0.05$; Fig. 7a) as well as between MeA *Kiss1* cells and LS *Kiss1* cells ($P < 0.05$; Fig. 7b). In contrast, there was no significant correlation between MeA and AVPV/PeN *Kiss1* expression (Fig. 7c). Thus, extra-hypothalamic *Kiss1* expression is upregulated similarly in each brain area (MeA, BnST, and LS) for a given animal, whereas AVPV/PeN *Kiss1* levels are not related to MeA levels.

Experiment 3: *Kiss1* is notably expressed in the MeA and BnST of $GABA_BR$ KO mice even in the absence of gonadal sex steroids

Under normal conditions (WT mice), *Kiss1* expression is basically undetectable in the MeA, BnST, and LS when gonadal sex steroids are absent. However, given that loss of $GABA_BR$ signaling strongly upregulates *Kiss1* in these areas in both gonad-intact and E_2-treated mice, we hypothesized that when GABA signaling is reduced, *Kiss1* may still be notably expressed in extra-hypothalamic regions even when sex steroids are absent. To assess this possibility, we examined *Kiss1* expression in the MeA, BnST, and LS of GDX $GABA_BR$ KO and WT mice that were not treated with any sex steroids. As expected, both WT mice and $GABA_BR$ KO mice had comparably elevated LH levels (data not shown), confirming the absence of sex steroid negative feedback. In the brain, we found that *Kiss1* expression in the MeA was virtually absent in GDX WT controls of each sex, as expected. In contrast, MeA *Kiss1* was readily detectable in GDX $GABA_BR$ KO mice and was significantly higher in these mice vs GDX WT mice ($P < 0.05$; Fig. 8). This outcome was true for $GABA_BR$ KOs of both sexes (Fig. 8), indicating that *Kiss1* expression in the MeA can be induced independently of E_2 stimulation. We found a similar pattern in the BnST: *Kiss1* expression in the BnST was significantly elevated in GDX $GABA_BR$ KO males, unlike in GDX WT males, which showed essentially absent levels as expected ($P < 0.05$; Fig. 9). However, there was no significant difference in BnST *Kiss1* expression between GDX $GABA_BR$ KO and

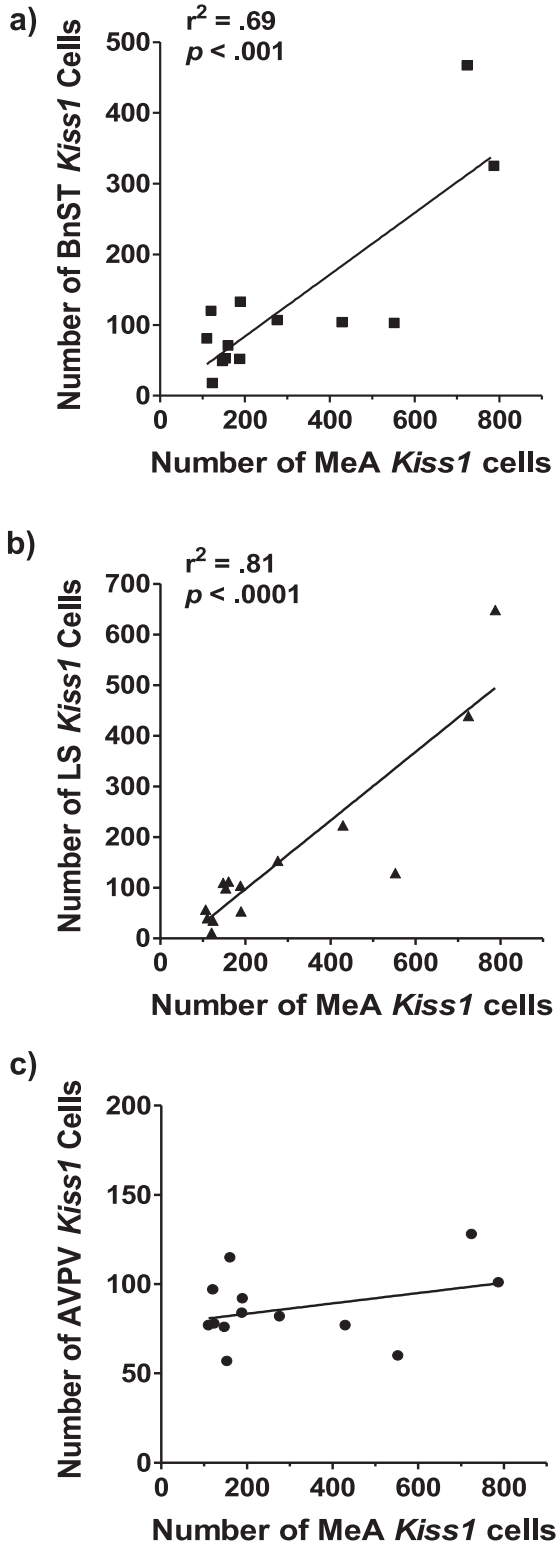

Figure 7. Regression analysis of MeA *Kiss1* expression and BnST, LS, and AVPV/PeN *Kiss1* expression in E_2-treated males, both WT and $GABA_BR$ KOs combined (n = 13 to 14 mice per brain region), showing that the number of MeA *Kiss1* cells is positively related ($P < 0.05$) to the number of (a) BnST and (b) LS *Kiss1* cells, but not the number of (c) AVPV/PeN *Kiss1* cells.

WT females (Fig. 9). In the LS, there were no significant differences in *Kiss1* expression levels between GDX $GABA_BR$ KO and WT mice, regardless of sex (Fig. 10). However, whereas all GDX WT mice had no detectable LS *Kiss1* cells, a subset (~40%) of GDX $GABA_BR$ KO mice had notable *Kiss1* expression despite the absence of sex steroids (Fig. 10). Thus, unlike GDX WT mice, some GDX $GABA_BR$ KO mice can show notable *Kiss1* expression in the LS. In contrast to the extra-hypothalamic sites, *Kiss1* expression in the AVPV/PeN and the ARC did not differ between GDX $GABA_BR$ KO and GDX WT mice (data not shown), again indicating that $GABA_BR$ signaling has no effect on hypothalamic *Kiss1* expression (15).

Discussion

Hypothalamic (AVPV/PeN and ARC) kisspeptin regulates the reproductive axis by stimulating GnRH release (1, 5, 6, 47–51), and, consequently, most kisspeptin research focuses on the role of hypothalamic kisspeptin cells. Although most *Kiss1* neurons are located in the hypothalamus, there are smaller *Kiss1* populations in extra-hypothalamic areas such as the MeA, BnST, and LS (5–16), brain areas whose numerous behavioral and physiological functions include, among other things, modulating reproductive physiology and behavior (17–23). However, very little is known about the regulation and function of these extra-hypothalamic *Kiss1* populations. The current study expands our knowledge of the regulation of the MeA, BnST, and LS *Kiss1* populations by focusing on their regulation by two signaling factors, E_2 and GABA. First, using several lines of ERKO and WT mice, we demonstrated that *Kiss1* expression in the BnST and LS is stimulated by E_2, as in the MeA and AVPV/PeN, and that this upregulation by E_2 occurs via ERα, but not ERβ, signaling pathways. Next, using WT and $GABA_BR$ KO mice, we determined that E_2 exposure results in more *Kiss1* cells in the MeA and LS in the absence of $GABA_BR$ signaling than when $GABA_BR$ signaling is functionally intact, suggesting that E_2 and GABA are additive in their modulation of extra-hypothalamic *Kiss1* expression. Next, we demonstrated that the removal of gonadal steroids does not completely suppress *Kiss1* expression in the MeA and BnST, and in some cases the LS, when $GABA_BR$ signaling is diminished. Thus, *Kiss1* expression in these regions is not entirely dependent on E_2, and GABA and E_2 regulate *Kiss1* independently (Fig. 11). Finally, we showed that there is a significant positive correlation between *Kiss1* levels in the MeA, BnST, and LS, but not between these regions and hypothalamic (AVPV/PeN) *Kiss1* levels. This suggests that the three extra-hypothalamic *Kiss1* populations may be under identical regulation that includes, but is not limited to, GABA and E_2.

Hypothalamic *Kiss1* expression is regulated by gonadal sex steroids, with E_2 acting via ERα to suppress

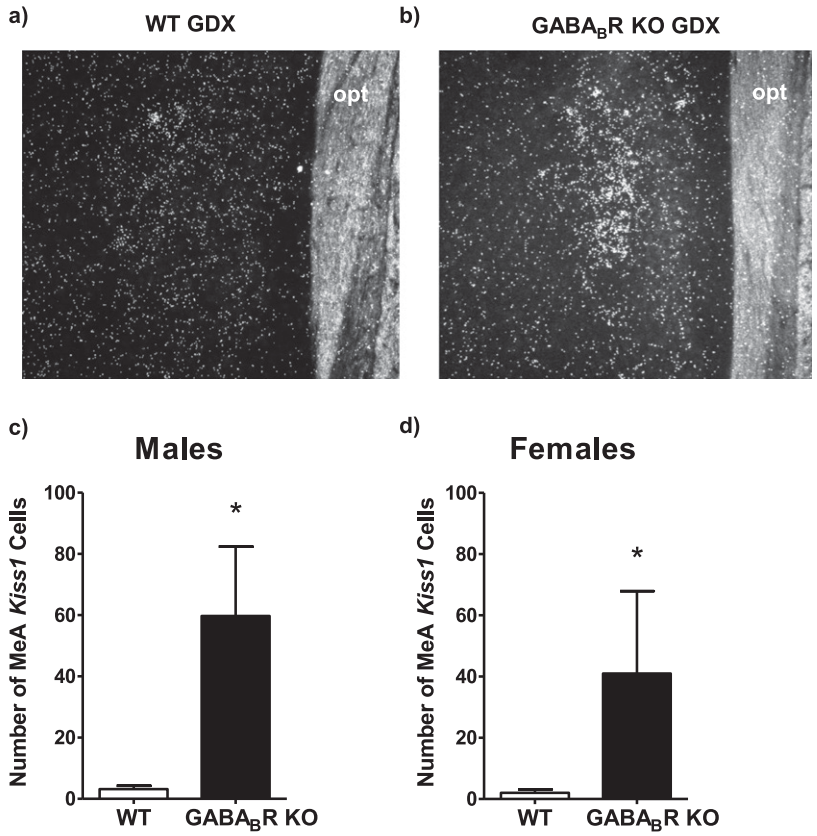

Figure 8. *Kiss1* mRNA expression in the MeA of GDX WT and GABA$_B$R KO mice. Representative images of MeA *Kiss1* expression in a (a) GDX WT and (b) GDX GABA$_B$R KO male mouse. The number of *Kiss1* cells in the MeA was significantly greater in GDX GABA$_B$R KO (c) males and (d) females in comparison with GDX WT mice (n = 6 to 9 mice per group). $^*P < 0.05$. opt, optic tract.

ARC *Kiss1* expression and upregulate AVPV/PeN *Kiss1* expression (8–10). *Kiss1* expression in the MeA, as in the AVPV/PeN, is also upregulated by E_2 via ERα (11, 31). However, it was previously unknown whether *Kiss1* in the BnST and LS is also regulated by E_2, whether this regulation is stimulatory or inhibitory, and whether it occurs via ERα or ERβ. Using WT, ERαKO, and βERKO mice, we determined that in the absence of circulating sex steroids (GDX), *Kiss1* expression in the BnST and LS was extremely low or totally absent regardless of genotype, consistent with extremely low *Kiss1* expression in the MeA of GDX mice (11, 31). In WT mice, E_2 treatment increased *Kiss1* expression in both the BnST and LS compared with GDX mice. Thus, all of the known extra-hypothalamic *Kiss1* populations (MeA, BnST, and LS) are similarly upregulated by E_2 signaling, which suggests that a primary function of *Kiss1* in these regions may be to mediate E_2 promotion of physiology and behavior. We found a similar E_2-induced upregulation of BnST and LS *Kiss1* in βERKO mice, indicating that ERβ is not necessary for the E_2 stimulation of *Kiss1* in these regions, despite the high presence of ERβ in the BnST. However, in E_2-treated ERαKO mice, *Kiss1* expression in both the BnST and LS remained low. Thus, ERα is required for E_2 upregulation of *Kiss1* expression in the BnST and LS, which is consistent with previous data showing that the MeA and hypothalamic *Kiss1* populations are primarily regulated by ERα (9, 10, 31).

In addition to E_2 upregulation, reduced GABA$_B$R signaling also drastically increases *Kiss1* expression in the MeA, BnST, and LS in gonad-intact mice (15). However, it was previously unknown whether E_2 exposure could further increase *Kiss1* expression in these areas in GABA$_B$R KO mice. In experiment 2, we found that MeA *Kiss1* expression was higher in E_2-treated GABA$_B$R KO mice compared with E_2-treated WT mice, indicating that even when *Kiss1* expression is elevated with E_2 treatment, diminished GABA$_B$R signaling can increase *Kiss1* expression even further. Therefore, E_2 and GABA$_B$R signaling independently regulate MeA *Kiss1* expression. Similarly, in the LS, we found that E_2-treated GABA$_B$R KO males had more *Kiss1* cells than did E_2-treated WT males, with a nonsignificant trend in the same direction for females. Thus, as in the MeA, absent GABA$_B$R signaling independently increases LS *Kiss1* expression above what is typically seen with E_2 treatment. In contrast to the MeA and LS, in the BnST, *Kiss1* expression did not differ between E_2-treated WT and GABA$_B$R KO mice, which suggests that E_2 treatment on its own maximizes BnST *Kiss1* expression. However, this conclusion should be tempered as it may reflect a statistical variability issue. Consistent with our previous data in gonad-intact mice (15), we found no difference in AVPV/PeN or ARC *Kiss1* expression between E_2-treated GABA$_B$R KO and WT mice, indicating that GABA$_B$R regulation of *Kiss1* is unique to extra-hypothalamic *Kiss1* populations.

Based on casual observance that within a given group, the mice with the highest MeA *Kiss1* levels often had very high BnST and LS levels, we also examined the relationship between the degree of MeA *Kiss1* expression and *Kiss1* expression levels in other brain regions. We found no significant relationship between the number of MeA *Kiss1* cells and the number of AVPV/PeN *Kiss1* cells. However, we did find significant positive relationships between the number of MeA *Kiss1* cells and both the number of BnST *Kiss1* cells and the

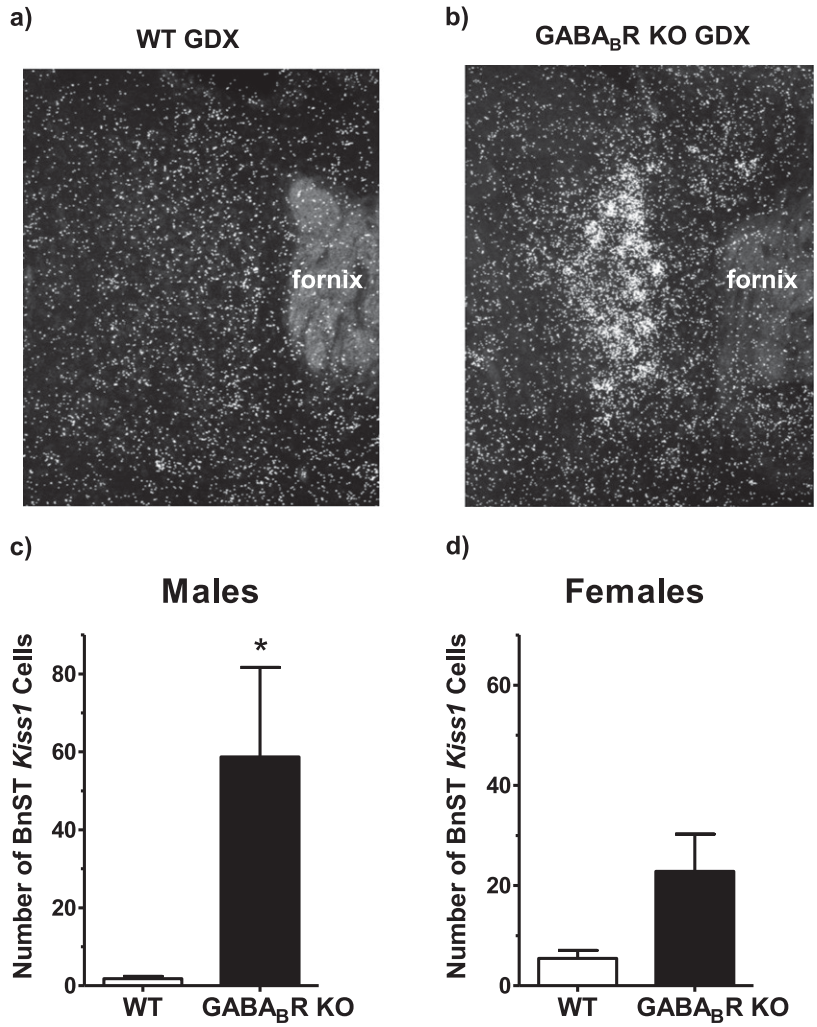

Figure 9. *Kiss1* mRNA expression in the BnST of GDX WT and GABA$_B$R KO mice. Representative images of BnST *Kiss1* expression in a (a) GDX WT and (b) GDX GABA$_B$R KO male mouse. (c) GDX GABA$_B$R KO males had more BnST *Kiss1* cells than did GDX WT males, whereas (d) GDX GABA$_B$R KO females had a comparable number of BnST *Kiss1* cells relative to GDX WT females (n = 6 to 9 mice per group). *$P < 0.05$.

number of LS *Kiss1* cells. This suggests that these extra-hypothalamic *Kiss1* populations are similarly regulated and that their expression levels tend to "track together." Thus, *Kiss1* levels in one extra-hypothalamic region, such as the MeA, can predict general levels in the same mouse in other extra-hypothalamic regions, such as the BnST and LS, but not the hypothalamus. Importantly, the very high *Kiss1* levels in the MeA, BnST, and LS of one animal vs moderate levels in another mouse from the exact same group suggests that an additional regulator besides E_2 and GABA can also influence extra-hypothalamic *Kiss1* levels. The identity of such additional *Kiss1* regulators, whether they be hormonal, neural, or epigenetic, as well as when during development/adulthood these factors regulate *Kiss1* neurons in the MeA, BnST, and LS remain unknown, but will be the focus of future investigations. Regardless, that *Kiss1* levels in all three extra-hypothalamic regions track with each other within any given animal suggests that they are being regulated similarly and may also indicate they are possibly all involved in a similar function.

Kiss1 expression in the MeA (11, 31) and in the BnST and LS (experiment 1) is usually very low or undetectable in the absence of gonadal steroids. However, because *Kiss1* in these three regions was greatly elevated in gonad-intact GABA$_B$R KO mice (15), we tested whether extra-hypothalamic *Kiss1* expression would still be undetectable in GDX GABA$_B$R KO mice as it is in GDX WT mice. In the MeA and BnST, we found that GDX GABA$_B$R KO mice had many detectable *Kiss1* cells, whereas GDX WT mice had almost no *Kiss1* cells. This indicates that *Kiss1* expression in the MeA and BnST can be induced by the removal of inhibitory GABA$_B$R signaling even when sex steroids are absent. Thus, the stimulatory effect of diminished GABA$_B$R signaling in these regions is independent of E_2 stimulation. In the LS, we found virtually no *Kiss1* cells in most GDX mice, regardless of genotype. However, unlike the GDX WT mice, which all had no LS *Kiss1* cells, a decent proportion (~40%) of GDX GABA$_B$R KO mice expressed a good amount (~40 to 50) of LS *Kiss1* cells. This bimodality in LS *Kiss1* expression of GDX GABA$_B$R KO mice again suggests that some other unknown regulator can differentially stimulate LS *Kiss1* expression when GABA$_B$R signaling is absent. However, when endogenous GABA$_B$R signaling is present (*e.g.*, WT mice), the inhibitory GABA signal appears to override any effects by other stimulatory neuropeptide/transmitters, such that all GDX WT mice fail to express LS *Kiss1*. Collectively, these findings indicate that in the absence of both gonadal sex steroids and GABA$_B$R signaling, the degree of increased *Kiss1* expression in extra-hypothalamic regions may indicate regional differences in the interaction of gonadal sex steroids and inhibitory GABA$_B$R signaling, as well as yet-to-be-identified additional regulators, on these extra-hypothalamic *Kiss1* populations.

Of note, these experiments used global ERKO and GABA$_B$R KO mice that are lacking ER or GABA$_B$R throughout both development and adulthood. It is

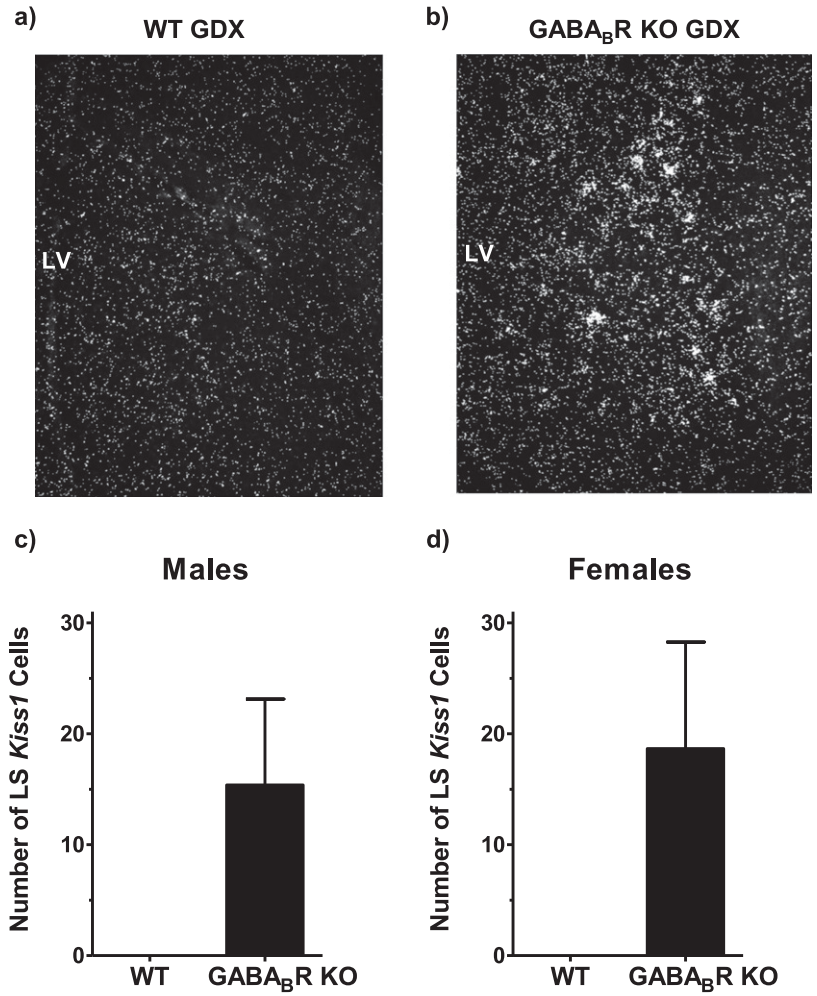

Figure 10. *Kiss1* mRNA expression in the LS of GDX WT and GABA$_B$R KO mice. Representative image of LS *Kiss1* expression in a (a) GDX WT and (b) GDX GABA$_B$R KO male. There was no difference in the mean number of LS *Kiss1* cells between GDX GABA$_B$R KO and GDX WT (c) males and (d) females (n = 6 to 9 mice per group). LV, lateral ventricle.

therefore unknown whether it is the absence of ER or GABA$_B$R signaling specifically in adulthood that alters *Kiss1* expression and/or whether the loss of this signaling during development may also permanently change these neurons or their afferents. MeA *Kiss1* expression is virtually absent in both WT and GABA$_B$R KO mice before puberty (15, 31), and BnST *Kiss1* expression is detected only at very low levels at prepubertal ages (15). Whether prepubertal/pubertal ER and/or GABA$_B$R signaling influences extra-hypothalamic *Kiss1* expression later in adulthood remains to be determined. Also, whereas our data indicate that both E_2 and GABA signaling can regulate extra-hypothalamic *Kiss1* expression, it is currently unknown whether these effects occur directly in *Kiss1* neurons or indirectly via "upstream" intermediary neurons. Approximately 65% of MeA *Kiss1* neurons express GABA$_B$R (15), indicating that GABA$_B$R signaling may possibly directly regulate MeA *Kiss1* neurons. However, it is currently unknown whether BnST and LS *Kiss1* neurons express GABA$_B$R or whether MeA, BnST, and LS *Kiss1* neurons express ERα, which would permit direct regulation by E_2.

Understanding how *Kiss1* neurons in the MeA, BnST, and LS are regulated and how this regulation differs between *Kiss1* populations may provide valuable insights regarding the reproductive and nonreproductive functions of these extra-hypothalamic *Kiss1* neurons. The MeA, BnST, and LS have numerous known effects on physiology and behavior, including reproductive endocrinology and sexual behavior. Lesions of the MeA disrupt ovarian cycles and prevent the E_2-mediated LH surge in females (18–20), whereas lesions of the BnST or the septum decrease LH levels and impair or enhance, respectively, aspects of sexual behavior (23, 27–30). Additionally, E_2 implanted directly into the MeA, BnST, or LS in diestrus rats advanced ovulation (25, 26), suggesting that these brain regions can modulate E_2-mediated gonadal sex steroid feedback. These previous findings, along with our findings that *Kiss1* levels in the MeA, BnST, and LS increase with E_2, suggest that one potential function of MeA, BnST, and LS kisspeptin may be to regulate reproductive physiology by influencing E_2-mediated feedback. Recent data also demonstrated that selective activation of MeA *Kiss1* neurons augmented sexual partner preference (52), which supports a possible role of MeA *Kiss1* neurons in mediating reproductive behaviors. Whether activation of BnST or LS neurons elicits similar behaviors has not yet been studied. Likewise, the functional implications of GABA inhibition of MeA, BnST, and LS *Kiss1* expression is not yet known and is the focus of new studies currently underway. Lastly, the current study and previous research (11, 15, 31) have found extra-hypothalamic *Kiss1* regulation to be similar between males and females when sex steroid levels are comparable, but whether sex differences in the functions of these extra-hypothalamic *Kiss1* neurons exist remains to be determined.

In conclusion, to our knowledge, our data are the first to demonstrate that *Kiss1* gene expression in the BnST and LS is upregulated by E_2 and that this occurs specifically via ERα. We also demonstrate that stimulatory E_2 and inhibitory GABA$_B$R signaling each independently

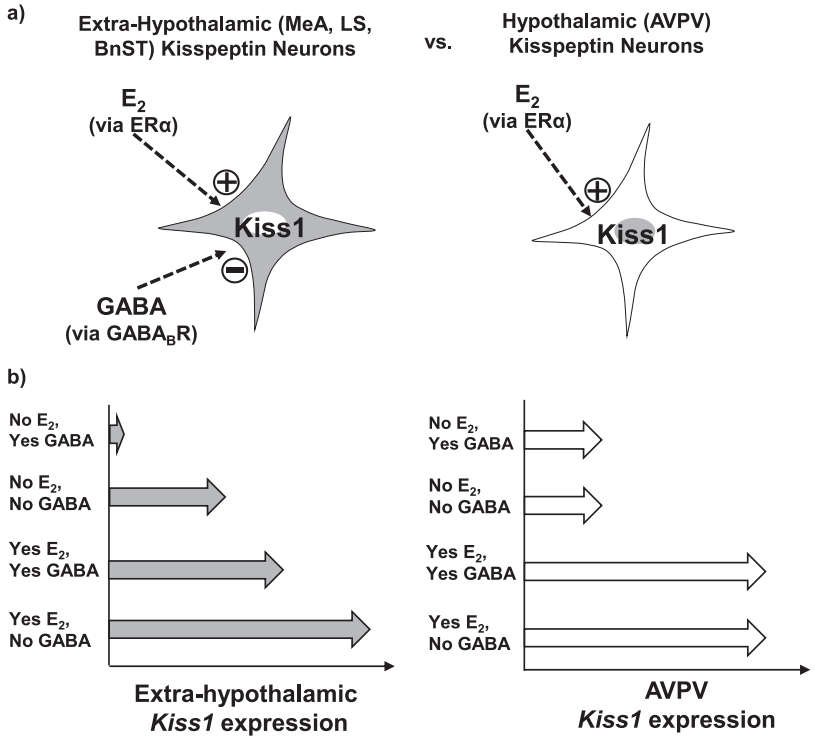

Figure 11. Cartoon schematic summarizing the regulation of extra-hypothalamic and AVPV/PeN hypothalamic *Kiss1* neurons. (a) E_2 stimulates both extra-hypothalamic and AVPV/PeN (hypothalamic) *Kiss1* neurons, whereas GABA inhibition, via $GABA_BR$, is unique to extra-hypothalamic *Kiss1* neurons. (b) General *Kiss1* expression patterns in extra-hypothalamic and hypothalamic (AVPV/PeN) areas with and without E_2 and $GABA_BR$ signaling, demonstrating that E_2 and GABA independently regulate extra-hypothalamic, but not hypothalamic, *Kiss1* levels.

regulate *Kiss1* in the MeA and other extra-hypothalamic regions, but that $GABA_BR$ signaling has no effect on hypothalamic *Kiss1* levels (Fig. 11). Along with showing that GABA's effects on extra-hypothalamic *Kiss1* do not rely on the gonadal sex steroid milieu, our findings also suggest that the degree of interactions of E_2 and $GABA_BR$ signaling on *Kiss1* expression may vary by brain region and that there may also be additional yet-to-be-identified regulatory factors besides GABA and E_2 that can stimulate *Kiss1* outside the hypothalamus.

Acknowledgments

Financial Support: This work was funded by National Institutes of Health Grants P50 HD012303 (to A.S.K.) and R01 HD090161 (to A.S.K.), as well as by Consejo Nacional de Investigaciones Científicas y Técnicas de Argentina PIP Grant 2013-571 (to V.A.L.-L.), Agencia Nacional de Promoción Científica y Tecnológica PICT Grant 2012 No. 707 (to V.A.L.-L.), and Agencia Nacional de Promoción Científica y Tecnológica PICT Grant 2015 No. 2795 (to N.P.D.G.). S.B.Z.S. was supported by National Institutes of Health Grants K99 HD092894, F32 HD088060, and T32 HD007203.

Correspondence: Alexander S. Kauffman, PhD, Department of Obstetrics, Gynecology, and Reproductive Sciences, University of California, San Diego, Leichtag Building, Room 3A-15, 9500 Gilman Drive, No. 0674, La Jolla, California 92093. E-mail: akauffman@ucsd.edu.

Disclosure Summary: The authors have nothing to disclose.

References

1. Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JS Jr, Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG, Zahn D, Dixon J, Kaiser UB, Slaugenhaupt SA, Gusella JF, O'Rahilly S, Carlton MB, Crowley WF Jr, Aparicio SA, Colledge WH. The *GPR54* gene as a regulator of puberty. *N Engl J Med*. 2003; **349**(17):1614–1627.
2. Topaloglu AK, Tello JA, Kotan LD, Ozbek MN, Yilmaz MB, Erdogan S, Gurbuz F, Temiz F, Millar RP, Yuksel B. Inactivating *KISS1* mutation and hypogonadotropic hypogonadism. *N Engl J Med*. 2012;**366**(7): 629–635.
3. Lapatto R, Pallais JC, Zhang D, Chan YM, Mahan A, Cerrato F, Le WW, Hoffman GE, Seminara SB. *Kiss1*$^{-/-}$ mice exhibit more variable hypogonadism than *Gpr54*$^{-/-}$ mice. *Endocrinology*. 2007;**148**(10): 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**(19):10972–10976.
5. Gottsch ML, Cunningham MJ, Smith JT, Popa SM, Acohido BV, Crowley WF, Seminara S, Clifton DK, Steiner RA. A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. *Endocrinology*. 2004;**145**(9):4073–4077.
6. 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**(6):2129–2134.
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**(3):225–230.
8. Kauffman AS, Gottsch ML, Roa J, Byquist AC, Crown A, Clifton DK, Hoffman GE, Steiner RA, Tena-Sempere M. Sexual differentiation of *Kiss1* gene expression in the brain of the rat. *Endocrinology*. 2007;**148**(4):1774–1783.
9. 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**(9):3686–3692.
10. Smith JT, Dungan HM, Stoll EA, Gottsch ML, Braun RE, Eacker SM, Clifton DK, Steiner RA. Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse. *Endocrinology*. 2005;**146**(7):2976–2984.
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**(5):2020–2030.
12. 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**(2):465–478.
13. Xu Z, Kaga S, Mochiduki A, Tsubomizu J, Adachi S, Sakai T, Inoue K, Adachi AA. Immunocytochemical localization of kisspeptin neurons in the rat forebrain with special reference to sexual

dimorphism and interaction with GnRH neurons. *Endocr J*. 2012; **59**(2):161–171.
14. Cravo RM, Margatho LO, Osborne-Lawrence S, Donato J Jr, Atkin S, Bookout AL, Rovinsky S, Frazão R, Lee CE, Gautron L, Zigman JM, Elias CF. Characterization of *Kiss1* neurons using transgenic mouse models. *Neuroscience*. 2011;**173**:37–56.
15. Di Giorgio NP, Semaan SJ, Kim J, López PV, Bettler B, Libertun C, Lux-Lantos VA, Kauffman AS. Impaired GABAB receptor signaling dramatically up-regulates *Kiss1* expression selectively in nonhypothalamic brain regions of adult but not prepubertal mice. *Endocrinology*. 2014;**155**(3):1033–1044.
16. Yeo SH, Kyle V, Morris PG, Jackman S, Sinnett-Smith LC, Schacker M, Chen C, Colledge WH. Visualisation of *Kiss1* neurone distribution using a Kiss1-CRE transgenic mouse. *J Neuroendocrinol*. 2016;**28**(11). doi: 10.1111/jne.12435.
17. Beltramino C, Taleisnik S. Facilitatory and inhibitory effects of electrochemical stimulation of the amygdala on the release of luteinizing hormone. *Brain Res*. 1978;**144**(1):95–107.
18. 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**(3):303–309.
19. Velasco ME, Taleisnik S. Effects of the interruption of amygdaloid and hippocampal afferents to the medial hypothalmus on gonadotrophin release. *J Endocrinol*. 1971;**51**(1):41–55.
20. 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**(4):927–934.
21. 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**(2): 554–559.
22. 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**(12):3541–3552.
23. Nance DM, Shryne J, Gorski RA. Septal lesions: effects on lordosis behavior and pattern of gonadotropin release. *Horm Behav*. 1974; **5**(1):73–81.
24. Carreras A, Mendoza C, Ortega E, Ruiz E. Testosterone implants into the lateral septum of male rats, a positive effect on LH and FSH secretion. *Brain Res Bull*. 1987;**19**(1):149–151.
25. Döcke F, Smollich A, Rhode W, Okrasa R, Dörner G. Studies on extrahypophyseal sites of estrogen action in the induction of ovulation in rats. *Endokrinologie*. 1975;**65**(3):274–287.
26. Kawakami M, Kimura F, Highchi T. Localization and mechanism of stimulatory feedback action of estrogen: effect of limbic forebrain implantation of estradiol benzoate on advancement of ovulation. *Endocrinol Jpn*. 1975;**22**(4):327–338.
27. Powers JB, Newman SW, Bergondy ML. MPOA and BNST lesions in male Syrian hamsters: differential effects on copulatory and chemoinvestigatory behaviors. *Behav Brain Res*. 1987;**23**(3): 181–195.
28. Valcourt RJ, Sachs BD. Penile reflexes and copulatory behavior in male rats following lesions in the bed nucleus of the stria terminalis. *Brain Res Bull*. 1979;**4**(1):131–133.
29. Beltramino C, Taleisnik S. Dual action of electrochemical stimulation of the bed nucleus of the stria terminalis on the release of LH. *Neuroendocrinology*. 1980;**30**(4):238–242.
30. Carreras A, Robles R, Ruiz E, Ortega E, Mendoza C, Osorio C. Role of the septum on LH, FSH and prolactin secretion in male rats. *Brain Res Bull*. 1984;**13**(2):339–342.
31. Stephens SB, Chahal N, Munaganuru N, Parra RA, Kauffman AS. Estrogen stimulation of *Kiss1* expression in the medial amygdala involves estrogen receptor-α but not estrogen receptor-β. *Endocrinology*. 2016;**157**(10):4021–4031.
32. Roa J, Vigo E, Castellano JM, Gaytan F, Navarro VM, Aguilar E, Dijcks FA, Ederveen AG, Pinilla L, van Noort PI, Tena-Sempere M. 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**(4):1627–1637.
33. Mitra SW, Hoskin E, Yudkovitz J, Pear L, Wilkinson HA, Hayashi S, Pfaff DW, Ogawa S, Rohrer SP, Schaeffer JM, McEwen BS, Alves SE. Immunolocalization of estrogen receptor β in the mouse brain: comparison with estrogen receptor α. *Endocrinology*. 2003; **144**(5):2055–2067.
34. 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**(4):507–525.
35. 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**(12):5267–5270.
36. Österlund 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**(1): 175–180.
37. Schuler V, Lüscher C, Blanchet C, Klix N, Sansig G, Klebs K, Schmutz M, Heid J, Gentry C, Urban L, Fox A, Spooren W, Jaton AL, Vigouret J, Pozza M, Kelly PH, Mosbacher J, Froestl W, Käslin E, Korn R, Bischoff S, Kaupmann K, van der Putten H, Bettler B. Epilepsy, hyperalgesia, impaired memory, and loss of pre- and postsynaptic $GABA_B$ responses in mice lacking $GABA_{B(1)}$. *Neuron*. 2001;**31**(1):47–58.
38. Bonaventura MM, Rodriguez D, Ferreira ML, Crivello M, Repetto EM, Bettler B, Libertun C, Lux-Lantos VA. Sex differences in insulin resistance in GABAB1 knockout mice. *Life Sci*. 2013;**92**(3):175–182.
39. Catalano PN, Bonaventura MM, Silveyra P, Bettler B, Libertun C, Lux-Lantos VA. $GABA_{B1}$ knockout mice reveal alterations in prolactin levels, gonadotropic axis, and reproductive function. *Neuroendocrinology*. 2005;**82**(5-6):294–305.
40. Catalano PN, Di Giorgio N, Bonaventura MM, Bettler B, Libertun C, Lux-Lantos VA. Lack of functional $GABA_B$ receptors alters GnRH physiology and sexual dimorphic expression of GnRH and GAD-67 in the brain. *Am J Physiol Endocrinol Metab*. 2010; **298**(3):E683–E696.
41. Di Giorgio NP, Catalano PN, López PV, González B, Semaan SJ, López GC, Kauffman AS, Rulli SB, Somoza GM, Bettler B, Libertun C, Lux-Lantos VA. Lack of functional GABAB receptors alters *Kiss1*, *Gnrh1* and *Gad1* mRNA expression in the medial basal hypothalamus at postnatal day 4. *Neuroendocrinology*. 2013; **98**(3):212–223.
42. Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P, Mark M. Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. *Development*. 2000;**127**(19):4277–4291.
43. 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.
44. Stephens SB, Tolson KP, Rouse ML Jr, Poling MC, Hashimoto-Partyka MK, Mellon PL, Kauffman AS. Absent progesterone signaling in kisspeptin neurons disrupts the LH surge and impairs fertility in female mice. *Endocrinology*. 2015;**156**(9):3091–3097.
45. Chowen JA, Argente J, Vician L, Clifton DK, Steiner RA. Proopiomelanocortin messenger RNA in hypothalamic neurons is increased by testosterone through aromatization to estradiol. *Neuroendocrinology*. 1990;**52**(6):581–588.
46. Navarro VM, Gottsch ML, Wu M, García-Galiano D, Hobbs SJ, Bosch MA, Pinilla L, Clifton DK, Dearth A, Ronnekleiv OK, Braun RE, Palmiter RD, Tena-Sempere M, Alreja M, Steiner RA. Regulation of NKB pathways and their roles in the control of Kiss1 neurons in the arcuate nucleus of the male mouse. *Endocrinology*. 2011;**152**(11):4265–4275.
47. Dhillo WS, Chaudhri OB, Patterson M, Thompson EL, Murphy KG, Badman MK, McGowan BM, Amber V, Patel S, Ghatei MA,

Bloom SR. Kisspeptin-54 stimulates the hypothalamic-pituitary gonadal axis in human males. *J Clin Endocrinol Metab*. 2005; **90**(12):6609–6615.

48. Navarro VM, Castellano JM, Fernández-Fernández R, Tovar S, Roa J, Mayen A, Nogueiras R, Vazquez MJ, Barreiro ML, Magni P, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M. Characterization of the potent luteinizing hormone-releasing activity of KiSS-1 peptide, the natural ligand of GPR54. *Endocrinology*. 2005; **146**(1):156–163.
49. Irwig MS, Fraley GS, Smith JT, Acohido BV, Popa SM, Cunningham MJ, Gottsch ML, Clifton DK, Steiner RA. Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat. *Neuroendocrinology*. 2004;**80**(4):264–272.
50. Messager S, Chatzidaki EE, Ma D, Hendrick AG, Zahn D, Dixon J, Thresher RR, Malinge I, Lomet D, Carlton MB, Colledge WH, Caraty A, Aparicio SA. Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. *Proc Natl Acad Sci USA*. 2005;**102**(5):1761–1766.
51. Navarro VM, Castellano JM, Fernández-Fernández R, Tovar S, Roa J, Mayen A, Barreiro ML, Casanueva FF, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M. Effects of KiSS-1 peptide, the natural ligand of GPR54, on follicle-stimulating hormone secretion in the rat. *Endocrinology*. 2005;**146**(4):1689–1697.
52. Adekunbi DA, Li XF, Lass G, Shetty K, Adegoke OA, Yeo SH, Colledge WH, Lightman SL, O'Byrne KT. Kisspeptin neurones in the posterodorsal medial amygdala modulate sexual partner preference and anxiety in male mice. *J Neuroendocrinol*. 2018; **30**(3):e12572. doi: 10.1111/jne.12572.