

Regulation of *Kiss1* Expression by Sex Steroids in the Amygdala of the Rat and Mouse

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Kisspeptin (encoded by the *Kiss1* gene) is an important regulator of reproduction. In rodents, *Kiss1* is expressed in two hypothalamic regions, the arcuate nucleus and anteroventral periventricular/periventricular continuum, where it is regulated by sex steroids. However, the distribution, regulation, and functional significance of neural kisspeptin outside of the hypothalamus have not been studied and are poorly understood. Here, we report the expression of *Kiss1* in the amygdala, predominantly in the medial nucleus of the amygdala (MeA), a region implicated in social and emotional behaviors as well as various aspects of reproduction. In gonadally intact rats and mice, *Kiss1*-expressing neurons were identified in the MeA of both sexes, with higher *Kiss1* expression levels in adult males than females in diestrus. In rats, *Kiss1* expression in the MeA changed as a function of the estrous cycle, with highest levels at proestrus. Next, we tested whether *Kiss1* in the MeA is regulated by the circulating sex steroid milieu. *Kiss1* levels in the MeA were low in gonadectomized mice and rats of both sexes, and treatment with either testosterone or estradiol amplified *Kiss1* expression in this region. Testosterone's inductive effect on *Kiss1* expression in the MeA likely occurs via estrogen receptor-dependent pathways, not through the androgen receptor, because dihydrotestosterone (a nonaromatizable androgen) did not affect MeA *Kiss1* levels. Thus, in rodents, *Kiss1* is expressed and regulated by sex steroids in the MeA of both sexes and may play a role in modulating reproduction or brain functions that extend beyond reproduction. (*Endocrinology* 152: 2020–2030, 2011)

Kisspeptin, encoded by the *Kiss1* gene, and its receptor (Kiss1R) play an essential role in controlling reproduction in mammals, based on several lines of evidence. First, humans and mice with mutations in either the *Kiss1R* or *Kiss1* gene exhibit striking deficits in reproductive function, including impaired pubertal maturation, low levels of sex steroids and gonadotropins, and infertility (1–4). Second, administration of kisspeptin robustly stimulates gonadotropin secretion (5, 6). Lastly, central administration of kisspeptin induces Fos expression in GnRH neurons, evokes depolarization and sustained firing of action potentials in these cells, and stimulates GnRH secretion (5, 6). In rodents, sheep, and primates (including

humans), neurons that express *Kiss1* mRNA and kisspeptin are located in two discrete regions of the diencephalon: the anteroventral periventricular nucleus-periventricular nucleus continuum (AVPV/PeN; or the preoptic area in sheep and primates) and, more caudally, the arcuate nucleus of the hypothalamus (ARC; the homolog of the primate infundibular nucleus) (7–12).

Although the regulation and function of *Kiss1* neurons in the hypothalamus has been well studied, the presence and importance of *Kiss1* neurons in other brain regions has been largely ignored. Virtually all roles of neural kisspeptin signaling in the control of puberty and reproduction have been attributed to one or both of the two hypo-

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Abbreviations: AR, Androgen receptor; ARC, arcuate nucleus of the hypothalamus; AVPV/PeN, anteroventral periventricular nucleus-periventricular nucleus continuum; DHT, dihydrotestosterone; E₂, estradiol; ER, estrogen receptor; GDH, gonadectomized; ISH, *in situ* hybridization; Kiss1R, Kiss1 receptor; MeA, medial nucleus of the amygdala; qPCR, quantitative real-time PCR; RNase, ribonuclease; SSC, sodium citrate, sodium chloride; T, testosterone.

thalamic populations, disregarding the possibility of kisspeptin signaling derived from potential *Kiss1* populations residing outside the hypothalamus. However, during the course of our earlier studies of *Kiss1* in the hypothalamus, we observed significant *Kiss1* expression in the amygdala. The amygdala is a limbic area rich in sex steroid receptors (13), which has previously been implicated in a wide range of sex steroid-dependent behavioral and physiological processes, including anxiety and fear responses, as well as social and sexual behaviors (14–19). Moreover, studies of the amygdala involving lesions, localized hormone treatments, and electrophysiology have implicated this region in the regulation of female reproductive cycles and sexual behavior (20–30). Additionally, the amygdala sends neuronal projections to target sites known to be involved in reproduction, including the preoptic area (where GnRH neurons reside), the AVPV, and ARC (31). There are also numerous documented sex differences in the amygdala, particularly in the medial nucleus, with males having greater medial amygdala nuclear volume, synaptic input, and dendritic spine density than females (32–36) and greater numbers of vasopressin- and substance P-producing neurons (14). Despite ample knowledge of the neuroanatomy and functions of the amygdala, at present, there is a complete paucity of information regarding *Kiss1* cells in this region.

In rodents, there are several key differences in the physiology and phenotype of *Kiss1* populations in the ARC and AVPV/PeN. First, sex steroids [e.g. estradiol (E_2) and testosterone (T)] regulate *Kiss1* gene expression in opposite fashions in these two hypothalamic areas. In the ARC, sex steroids inhibit the expression of *Kiss1*, whereas in the AVPV/PeN, sex steroids increase *Kiss1* expression (37–39). Conversely, when sex steroids are low, *Kiss1* expression increases in the ARC and decreases in the AVPV/PeN (37–39). Second, in the ARC, sex steroids inhibit *Kiss1* expression via either androgen receptors (AR) or estrogen receptors (ER), whereas in the AVPV/PeN, sex steroids induce *Kiss1* expression only through ER-dependent pathways (38, 39). Third, the cellular phenotype of *Kiss1* neurons differs between the two regions. In the ARC, but not the AVPV/PeN, *Kiss1* neurons coexpress dynorphin, neurokinin B, and the neurokinin B receptor NK3R (40, 41). Conversely, in the AVPV/PeN, but not the ARC, *Kiss1* neurons coexpress tyrosine hydroxylase (42). Finally, *Kiss1* neurons in the AVPV/PeN are sexually dimorphic, with females having more *Kiss1* neurons than males, whereas *Kiss1* cell number in the ARC is not demonstrably sexually dimorphic in adults under controlled sex steroid conditions (37, 42, 43). Collectively, these findings emphasize that *Kiss1* neurons in different brain regions are phenotypically and physiologically distinct, likely relating to differences in function (5, 6). Whether *Kiss1* cells else-

where in the brain, such as the amygdala, share common properties with the AVPV/PeN and/or ARC population is unknown.

Here, we investigated the pattern and regulation of *Kiss1* expression in the amygdala of two rodent species, the rat and mouse. We had four main goals: 1) to determine whether *Kiss1* is significantly expressed in the amygdala in each species and, if so, precisely where in this region; 2) to assess whether *Kiss1* expression in the amygdala of adult animals is sexually dimorphic, as it is in the AVPV/PeN; 3) to determine whether *Kiss1* neurons in the amygdala, like those in the hypothalamus, are targets for regulation by sex steroids and, if so, whether *Kiss1* expression in the amygdala is stimulated or inhibited by sex steroids; and 4) to elucidate which sex steroid receptor pathway, AR and/or ER, mediates any effects of sex steroids on *Kiss1* expression in the amygdala.

Materials and Methods

Animals

Male and female C57BL6 mice were housed at the University of California, San Diego, and University of Washington on a 12-h light, 12-h dark cycle with food and water available *ad libitum*. Rat brain tissue was used from adult Wistar rats of both sexes that were used in previous reports by our labs (37, 44). All experiments were conducted in accordance with the National Institutes of Health Animal Care and Use Guidelines and with approval of the local Animal Care and Use Committees of the University of California, San Diego, and the University of Washington.

Surgical treatments and tissue collection

Sex steroids up-regulate *Kiss1* gene expression in the AVPV/PeN and down-regulate *Kiss1* in the ARC (37, 38) and hence could also augment *Kiss1* expression in other brain regions. Thus, in some experiments, circulating sex steroid levels [E_2 , T, or dihydrotestosterone (DHT)] were equalized among groups using SILASTIC (Dow Corning Corp., Midland, MI) implants. Adults of both sexes were anesthetized and bilaterally gonadectomized (GDX). At the time of gonadectomy, animals received a sc E_2 -filled, T-filled, or DHT-filled SILASTIC implant, constructed as previously described (37, 39). For rats, the E_2 , T, and DHT implants have been shown to successfully reduce circulating LH and FSH levels in GDX animals to levels found in intact animals and to also significantly regulate hypothalamic *Kiss1* expression (37). For mice, the DHT and T implants have been shown to produce physiological levels of hormone (~3 and 11 ng/ml, respectively) similar to levels in intact males, and the E_2 implants produce slightly elevated levels around 50–88 pg/ml (39, 45); all three implants have been shown to regulate *Kiss1* expression in the murine hypothalamus (39). For comparison, some GDX animals of each sex received no hormone implant. One week after surgery, all animals were killed via rapid decapitation. To avoid circadian modulation of *Kiss1* expression (46), all animals were killed in the late morning/early afternoon. At the

time of killing, brains were collected, immediately frozen on dry ice, and stored at -80°C .

Quantitative (real-time) PCR analysis of *Kiss1* gene expression

In one experiment, total RNA from 600- μm -thick frozen micropunches of the medial nucleus of the amygdala (MeA) and AVPV/PeN was extracted using the RNeasy Lipid Tissue Mini kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. RNA was reverse transcribed using the Omniscript RT kit (QIAGEN), and cDNA was stored at -20°C until use in PCR. Quantitative real-time PCR (qPCR) was performed on each cDNA sample using the Bio-Rad iCycler Detection System and Quantitect SYBR Green PCR kit (QIAGEN). To detect *Kiss1*, *Kiss1*-specific primers 5'-CAA AAG TGA AGC CTG GAT CC-3' (forward) and 5'-GTT GTA GGT GGA CAG GTC C-3' (reverse) were used. Standard curves were generated for each product using cloned cDNAs for *Kiss1* and *GAPDH* to quantify the abundance of cDNA in each sample. For standard curves, a dilution series of cloned *Kiss1* and *GAPDH* templates ranging from 10 to 10^8 copies were used. The quantitative PCR cycling parameters were one cycle of 95°C for 15 min, followed by 40 cycles of 94°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec. Data collection was taken at the 72°C extension phase. To ensure the presence of a single product, a dissociation curve was performed after each run. Data were collected from threshold values using the automatic function of the Bio-Rad MyIQ software. All samples were run in duplicate, and *Kiss1* values were normalized to *GAPDH*, whose expression is constant.

Single-label *in situ* hybridization (ISH)

Single-label ISH for *Kiss1* was performed as previously described (11, 37, 39, 46), using a validated *Kiss1* riboprobe (11). Briefly, five alternating sets of 20- μm brain sections (*i.e.* each set contains every fifth section) encompassing the entire forebrain and hypothalamus/amygdala regions were cut and thaw-mounted onto SuperFrost Plus slides and stored at -80°C . One set of sections spanning the entire MeA (or AVPV/PeN or ARC) was fixed in 4% paraformaldehyde, pretreated with acetic anhydride, rinsed in $2\times$ sodium citrate, sodium chloride (SSC), delipidated in chloroform, dehydrated in ethanols, and air dried. Radiolabeled (^{33}P) antisense riboprobe (0.05 pmol/ml) was combined with 1/20 volume yeast tRNA (Roche Biochemicals, Indianapolis, IN), heat denatured, added to hybridization buffer, and applied to each slide (100 μl /slide). Slides were coverslipped and put at 55°C for 17 h. The next day, the slides were washed in $4\times$ SSC and placed into ribonuclease (RNase) [37 mg/ml RNase A in 0.15 M sodium chloride, 10 mM Tris, 1 mM EDTA (pH 8.0)] for 30 min at 37°C , then in RNase buffer without RNase at 37°C for 30 min. After washing in $2\times$ SSC at room temperature, slides were washed in $0.1\times$ SSC at 62°C , dehydrated in ethanols, and air dried. Slides were then dipped in Kodak NTB emulsion, air dried, and stored at 4°C for 7–12 d (depending on the assay). Slides were then developed, dehydrated in ethanols, cleared in Citrisolv (Fisher Scientific, Pittsburgh, PA), and coverslipped with Permaslip (Sigma Chemical Co., St. Louis, MO). Slides were analyzed with an automated image processing system and custom grain counting software (Dr. Don Clifton, University of Washington) by a person blind to the treatment group. The software counts the number of silver grain clusters representing *Kiss1* cells as well as the number of

silver grains in each cell cluster (a semiquantitative index of mRNA content per cell) (38, 47). Cells were considered *Kiss1* positive when the number of silver grains in a cluster exceeded that of background by 3-fold.

Statistical analyses

All data are expressed as the mean \pm SEM for each group. Differences in group means were assessed via overall ANOVA, with *post hoc* analysis determined by Fisher's least significant difference test. For all comparisons, statistical significance was set at $P < 0.05$. All analyses were performed with Statview version 5.0.1 (SAS Institute, Cary, NC).

Experiment 1: *Kiss1* gene expression in the MeA of intact male and female rats

Experiment 1 mapped the specific neuroanatomical expression of *Kiss1* mRNA outside the hypothalamus, particularly in the amygdala region, in gonadally intact adult male and female rats ($n = 5$ per sex). Adult females were cycled via examination of daily vaginal smears and brains collected at diestrous stage. Single-label ISH was used to identify and compare *Kiss1* mRNA expression in specific regions of the amygdala in each sex. The mean number of *Kiss1*-expressing cells and the relative level of *Kiss1* mRNA per cell (as reflected by silver grains per cell) were determined.

Experiment 2: *Kiss1* expression in the MeA of female rats across the estrous cycle

Experiment 1 detected low levels of *Kiss1* expression in the MeA of female rats on diestrus. We previously reported that *Kiss1* levels in the AVPV/PeN and ARC of rats change with the estrous cycle (44). Thus, this experiment tested whether *Kiss1* expression in the MeA also changes over the estrous cycle. Brain tissue was used from a previous study (44) in which female rats were cycled by daily inspection of vaginal smears. After displaying two continuous cycles, females were killed in the morning on either diestrus II, proestrus, or estrus ($n = 5$ per group). Brains were collected and analyzed via ISH for the number of *Kiss1*-expressing cells in the MeA as well as the relative amount of *Kiss1* mRNA per cell in this region.

Experiment 3: sex steroid regulation of *Kiss1* expression in the MeA of male and female rats

In the rodent hypothalamus, sex steroids (T and E_2) stimulate *Kiss1* levels in the AVPV/PeN and inhibit *Kiss1* in the ARC. This experiment tested whether *Kiss1* gene expression is also regulated by sex steroids in the MeA and, if so, in which direction (stimulatory as in the AVPV/PeN or inhibitory as in the ARC). Adult male and female rats were GDX and treated for a week with either E_2 or nothing ($n = 5$ per group). To compare the effects of T and E_2 , an additional cohort of five GDX males received a T implant for 1 wk. After 1 wk, all animals were killed and the brains collected. Sections encompassing the amygdala were processed for *Kiss1* ISH to determine the number of *Kiss1*-expressing cells as well as the relative amount of *Kiss1* mRNA per cell.

Experiment 4: *Kiss1* expression in the MeA of male and female mice

The previous experiments identified *Kiss1* in the MeA of rats, with higher expression levels in adult males than females on diestrus. We next determined whether the presence of *Kiss1* in the

MeA in rats also extends to other rodent species. In this experiment, we assessed whether *Kiss1* is expressed in the MeA of male and female mice and whether there is a sex difference in MeA *Kiss1* levels between gonadally intact mice, as there was in rats. Adult male and female (diestrus) mice were killed and the brains collected and processed for *Kiss1* ISH, as in experiment 1 ($n = 5$ –6 per sex).

Experiment 5: E_2 regulation of *Kiss1* expression in the MeA of mice

As in rats, *Kiss1* expression in the AVPV/PeN and ARC of mice is strongly regulated by sex steroids, with T and E_2 stimulating *Kiss1* in the AVPV/PeN and inhibiting *Kiss1* in the ARC. Given the robust sex steroid regulation we observed in the MeA of rats (experiment 3), we tested whether *Kiss1* in the MeA of mice is also stimulated by sex steroids, particularly E_2 . Adult male and female mice were GDX and treated for a week with E_2 or nothing ($n = 5$ –7 per group). Mice were then killed and the brains collected for ISH of *Kiss1* levels in the MeA. To confirm data obtained with ISH, we also conducted a similar experiment in a separate cohort of mice ($n = 5$ per group) whose brains were analyzed for *Kiss1* levels using the sensitive and quantitative method of real-time PCR (qPCR). For each animal, a micropunch was taken from a 600- μ m brain section that included the MeA region. For comparison, similar-sized micropunches were also taken from the AVPV/PeN and olfactory bulb regions. Total mRNA was extracted from the micropunches and qPCR for *Kiss1* performed on cDNA obtained via reverse transcription.

Experiment 6: effects of androgens on *Kiss1* in the MeA of mice

In experiment 3, we determined that both E_2 and T can stimulate *Kiss1* in the male rat MeA. Here we tested whether T can also stimulate *Kiss1* in the MeA in mice, in either males or females. In experiment 6A, adult male and female mice were GDX and treated for a week with T or nothing ($n = 4$ per group). Animals were then killed and the brains collected for ISH of *Kiss1* levels in the MeA. Because T can act via AR or via ER (after aromatization to E_2), it is unclear which pathway mediates T's stimulation of *Kiss1* in the MeA. Thus, in experiment 6B, we tested whether the *Kiss1* gene in the MeA can be regulated selectively via AR pathways. GDX adult male mice were given an implant containing DHT (a nonaromatizable androgen), E_2 , or nothing ($n = 4$ –6 per group). After 1 wk, mice were killed and the brains collected and processed via ISH to determine the number of *Kiss1*-expressing cells in the MeA. For comparison of the effects of the different hormones, *Kiss1* levels were also determined in the AVPV/PeN and the ARC.

Results

Experiment 1: the *Kiss1* gene is expressed in the MeA of gonadally intact male and female rats

This experiment mapped the expression of *Kiss1* mRNA expression in the amygdala of gonadally intact male and female (diestrus) rats. We observed distinct clusters of silver grains (reflecting *Kiss1* mRNA staining) scattered in the amygdala of both sexes, although levels were more noticeable in males (Fig. 1). *Kiss1* neurons seemed to be restricted

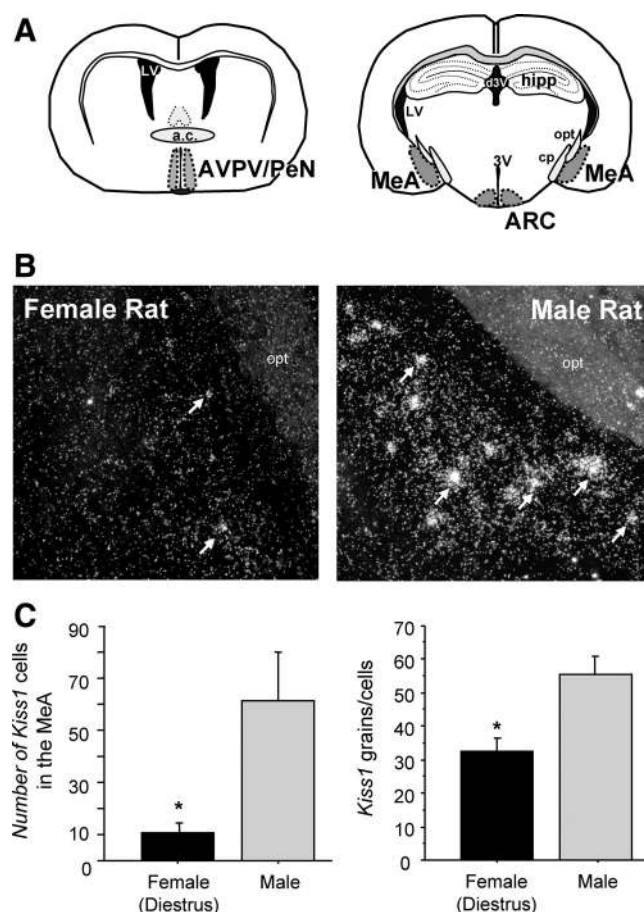


FIG. 1. *Kiss1* mRNA expression in the amygdala of rats. A, Schematic of the different brain regions where *Kiss1* expression is identified. a.c., Anterior commissure; hipp, hippocampus; LV, lateral ventricle; opt, optic tract; pd, cerebral peduncle; 3V, third ventricle; d3V, dorsal 3rd ventricle. B, Representative photomicrographs of ISH for *Kiss1* mRNA in the MeA of gonadally intact male and female rats. White arrows depict sample *Kiss1* neurons. C, Mean number of *Kiss1* neurons in the MeA of gonadally intact female and male rats. The number of *Kiss1* neurons was significantly higher in males than females (diestrus). *, Significantly different ($P < 0.05$).

to the MeA and were not observed in other amygdaloid regions. Although *Kiss1* cells were most prominent in the posterodorsal MeA, there were also occasional cells in more ventral regions of the MeA. Quantitative analysis revealed that male rats had significantly more *Kiss1* neurons in the MeA than did diestrus females ($P < 0.05$, Fig. 1). The relative amount of *Kiss1* mRNA per cell was also higher in males than diestrus females ($P < 0.05$, Fig. 1).

Experiment 2: *Kiss1* expression in the MeA of female rats changes across the estrous cycle

Kiss1 levels in the AVPV/PeN and the ARC have previously been reported to change with the estrous cycle, and we tested whether the expression of *Kiss1* in the MeA also changes with estrous stage. We found that *Kiss1* expression was detectable in the MeA of female rats on diestrus, proestrus, and estrus but varied as a function of cycle

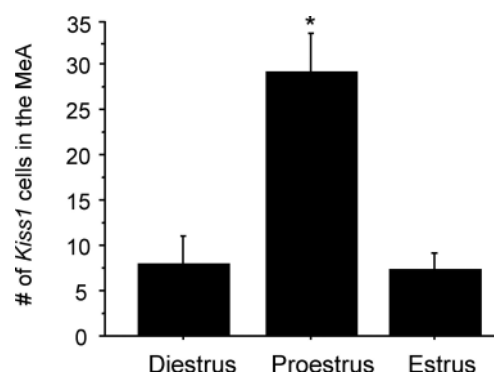


FIG. 2. Mean number of *Kiss1* cells in the MeA of female rats at different stages of the estrous cycle. *Kiss1* mRNA was significantly higher in females on proestrus than diestrus or estrus. *, Significantly different from other two groups ($P < 0.05$).

stage. Specifically, the number of *Kiss1* neurons in the MeA was highest on proestrus and lowest on diestrus and estrus ($P < 0.05$, Fig. 2). There was no significant difference in *Kiss1* cell numbers between estrus and diestrus. Correlating with *Kiss1* cell numbers in the MeA, mean plasma levels of E_2 were highest at proestrus (~ 55 pg/ml)

and low on diestrus and estrus (~ 16 – 23 pg/ml), as previously reported (44). No significant difference in the content of *Kiss1* mRNA per cell (as reflected by grains per cell) was detectable in the MeA during different stages of the estrous cycle (data not shown).

Experiment 3: sex steroids regulate *Kiss1* expression in the MeA of male and female rats

This experiment tested whether the *Kiss1* gene in the MeA is regulated by sex steroids and, if so, in which direction (stimulatory as in the AVPV/PeN or inhibitory as in the ARC). We found that sex steroid treatment induced *Kiss1* expression in the MeA of rats. GDX male and female rats each had low, sometimes undetectable, levels of *Kiss1* in the MeA (Fig. 3). In contrast, females and males given an E_2 implant for 1 wk had high *Kiss1* levels in the MeA ($P < 0.05$ vs. GDX animals, Fig. 3). An additional group of GDX males treated with T for 1 wk also had high levels of *Kiss1* in the MeA, which were not significantly different from those of E_2 -treated males or females (Fig. 3).

Experiment 4: *Kiss1* is expressed in a sexually dimorphic pattern in the MeA of gonadally intact mice

The previous experiments identified *Kiss1* in the MeA of rats of both sexes. Here, we examined the presence of *Kiss1* in the amygdala in gonadally intact male and female mice. We observed distinct *Kiss1*-expressing cells scattered loosely in the MeA of both sexes of mice. In mice, as in rats, *Kiss1* expression was greater in males than females on diestrus (Fig. 4) and were similarly restricted to the MeA region of the amygdaloid complex. Quantitative analysis determined that male mice had greater numbers of *Kiss1* neurons in the MeA than females on diestrus ($P < 0.05$, Fig. 4). The relative amount of *Kiss1* mRNA per cell trended toward being higher in males than diestrus females, but this did not reach statistical significance.

Experiment 5: E_2 up-regulates *Kiss1* expression levels in the MeA of mice

In mice, like rats, sex steroids stimulate the expression of *Kiss1* in the AVPV/PeN and inhibit *Kiss1* in the ARC. This experiment tested whether *Kiss1* in the MeA of mice is also regu-

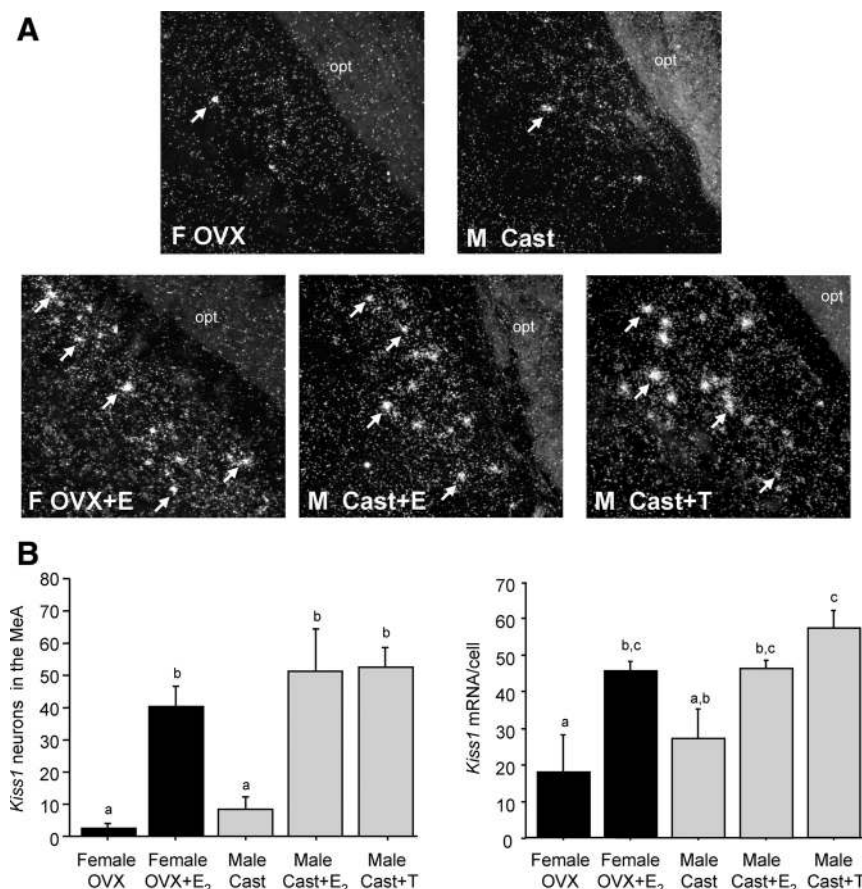


FIG. 3. A, Representative photomicrographs of *Kiss1* in the MeA of male and female rats treated with sex steroids for 1 wk. White arrows depict examples of *Kiss1* neurons. B, Mean number of *Kiss1* cells in the MeA of male and female rats that were GDX and treated with T, E_2 , or nothing. There were more *Kiss1* cells in the MeA in T- or E_2 -treated rats compared with untreated GDX rats. Bars with different letters are significantly different from each other ($P < 0.05$). Cast, Castrated; OVX, ovariectomized.

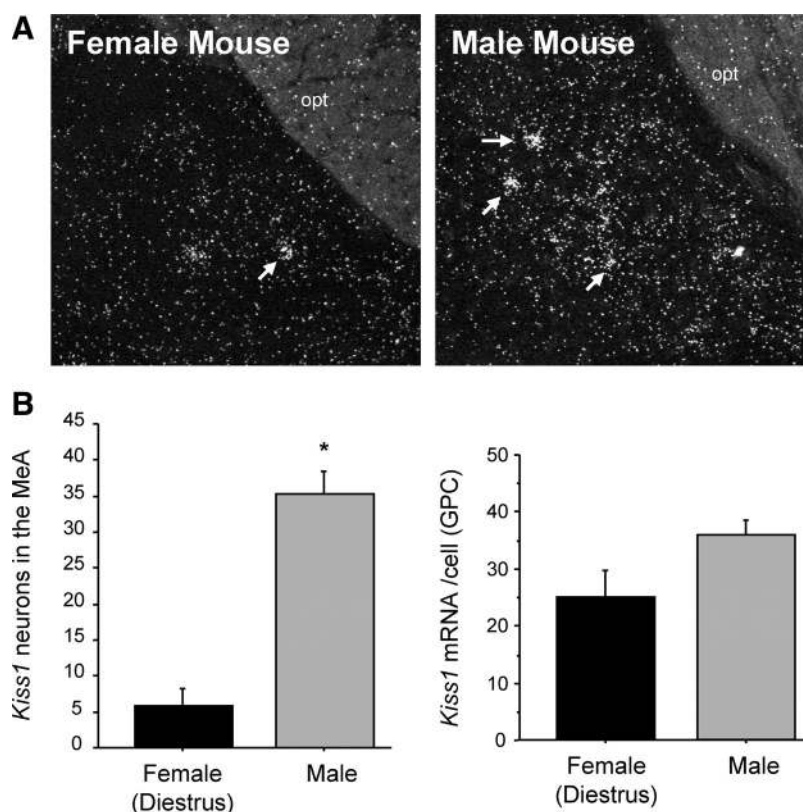


FIG. 4. *Kiss1* mRNA expression in the medial amygdala of male and female mice. **A**, Representative photomicrographs of ISH for *Kiss1* mRNA in the MeA of gonadally intact male and female (on diestrus) mice. White arrows depict examples of *Kiss1* neurons. opt, Optic tract. **B**, Mean number of *Kiss1* neurons in the MeA of gonadally intact female and male mice. The number of *Kiss1* neurons was significantly higher in males than females (on diestrus). GPC, Grains per cell. *, Significantly different ($P < 0.05$).

lated by sex steroids, specifically E_2 . First, using ISH, we determined that E_2 stimulates *Kiss1* levels in the murine MeA. Male and female mice that were GDX and given E_2 for a week had significantly more *Kiss1*-expressing cells in the MeA than untreated GDX mice ($P < 0.05$ for each sex, Fig. 5). The number of *Kiss1* cells in the MeA was not different between E_2 -treated males and E_2 -treated females. Although there was also a trend toward higher *Kiss1* mRNA content per cell in the MeA in E_2 -treated animals *vs.* GDX animals, this did not reach statistical significance (data not shown). To confirm the ISH findings, we repeated the experiment in a separate cohort of mice and used the sensitive method of qPCR to measure *Kiss1* mRNA levels in the MeA. We detected *Kiss1* mRNA in the MeA of all animals. In corroboration of the ISH data, we found that animals of each sex given E_2 had significantly more *Kiss1* mRNA in the MeA than GDX animals without E_2 (Fig. 6). A similar pattern of enhanced *Kiss1* expression in E_2 -treated animals was observed in the AVPV/PeN, where sex steroids have previously been shown to increase *Kiss1* levels (Fig. 6). Virtually no *Kiss1* mRNA was detected in the olfactory bulb (Fig. 6).

Experiment 6: T, but not a nonaromatizable androgen, stimulates *Kiss1* expression in the MeA of mice

In experiment 3, we determined that T can stimulate *Kiss1* in the MeA of male rats. Here we tested whether T can also regulate *Kiss1* in the MeA of mice of either sex. We determined that T significantly up-regulates *Kiss1* expression in the murine MeA in both sexes; GDX male and female mice given T implants had significantly more *Kiss1* neurons in the MeA than untreated GDX mice ($P < 0.05$, Fig. 7). This stimulatory effect of T was similar between the sexes.

Because T can act via either AR or ER (after aromatization to E_2), we tested whether MeA *Kiss1* expression can be induced by a nonaromatizable androgen (DHT), which can act only via AR. Although we found that GDX mice treated with E_2 had significantly elevated MeA *Kiss1* levels relative to untreated GDX mice ($P < 0.05$), DHT-treated mice did not have increased *Kiss1* expression in the MeA (Fig. 8). Levels of *Kiss1* in the MeA were not significantly different between untreated and DHT-treated mice and were low in each group (Fig. 8). A similar pattern was found in the AVPV/PeN, where E_2 but not DHT stimulated *Kiss1* levels ($P < 0.05$, Fig. 8). In contrast, both E_2 and DHT treatment significantly reduced *Kiss1* levels in the ARC relative to the high expression levels in untreated GDX mice in this region ($P < 0.05$ for both hormones, Fig. 8).

Discussion

After the discovery in 2003 that humans and mice lacking KISS1R exhibit hypogonadotropic hypogonadism and impaired puberty onset (1, 4), many reports testified to the functional significance of kisspeptin signaling in the brain to regulate mammalian reproduction. In virtually every instance, attention has been focused exclusively on *Kiss1* or kisspeptin neurons in the hypothalamus, specifically in the AVPV/PeN (or preoptic area in some species) and the ARC. However, we show here in the mouse and rat that *Kiss1* is also expressed outside the hypothalamus in an important limbic region, the amygdala, and more specifically, the MeA. Moreover, we demonstrate that *Kiss1* neurons in the MeA are regulated by the circulating levels of sex steroids in both sexes and that this hormonal regulation likely occurs via ER-dependent mechanisms rather than AR-dependent pathways. We also show that *Kiss1*

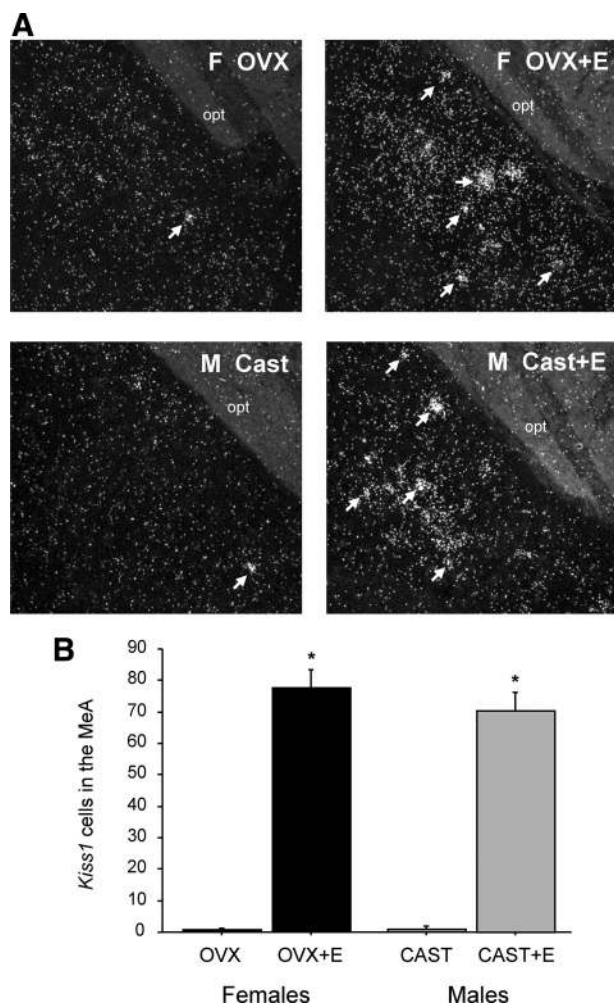


FIG. 5. A, Representative photomicrographs of *Kiss1* in the MeA of male and female mice treated with E_2 for 1 wk. B, Mean number of *Kiss1* cells in the MeA of male and female mice that were GDX and treated with E_2 . In each sex, there were more *Kiss1* cells in the MeA in E_2 -treated mice compared with untreated GDX mice. *, Significantly different ($P < 0.05$) from GDX control in same sex. Cast, Castrated; opt, optic tract; OVX, ovariectomized.

expression in the MeA is lower in gonadally intact females than males but that *Kiss1* levels change as a function of the estrous cycle in females, peaking at proestrus, suggesting a function relating to ovulation or sexual behavior. Collectively, these findings implicate a novel population of extrahypothalamic *Kiss1*-expressing neurons in participating in sex-steroid-regulated physiology and/or behavior.

In gonadally intact adult mice and rats of both sexes, we identified *Kiss1* mRNA-expressing cells in the amygdala, in addition to previously well-recognized areas of expression (AVPV/PeN and ARC) (37) (this report). In the amygdala, *Kiss1* neurons were primarily restricted to the MeA, most prominently the posterodorsal MeA and were not readily observed in other amygdaloid regions. Interestingly, in both mice and rats, *Kiss1*-expressing cells in the MeA were more readily detectable in gonadally intact

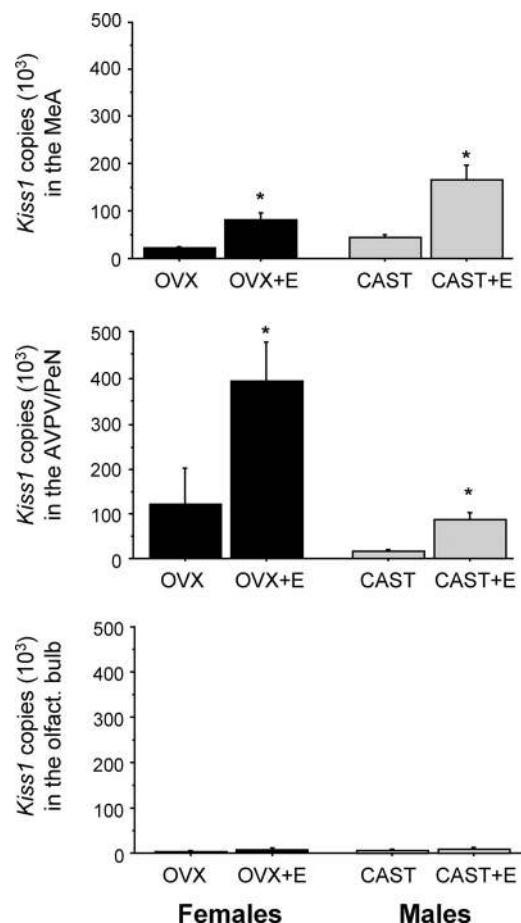


FIG. 6. Mean *Kiss1* mRNA copy number, as determined via qPCR, in the MeA and AVPV of male and female mice that were GDX and treated with E_2 or nothing. In both regions, there were significantly more *Kiss1* mRNA-expressing cells in E_2 -treated mice compared with untreated GDX mice. *, Significantly different ($P < 0.05$) from GDX mice in same region. Cast, Castrated; OVX, ovariectomized.

males than gonadally intact females on diestrus. Thus, like the *Kiss1* population in the AVPV/PeN, *Kiss1* expression in the MeA is sexually dimorphic in gonadally intact adult rodents, at least when females are in diestrus. Notably, the

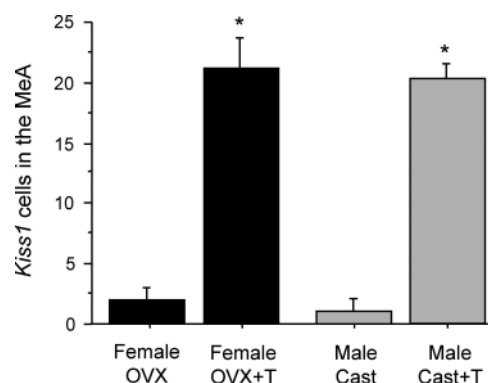


FIG. 7. Mean number of *Kiss1* cells in the MeA of male and female mice that were GDX and treated with T. In both sexes, there were more *Kiss1* cells in the MeA in T-treated mice compared with untreated GDX mice. *, Significantly different ($P < 0.05$) from GDX control in same sex. Cast, Castrated; OVX, ovariectomized.

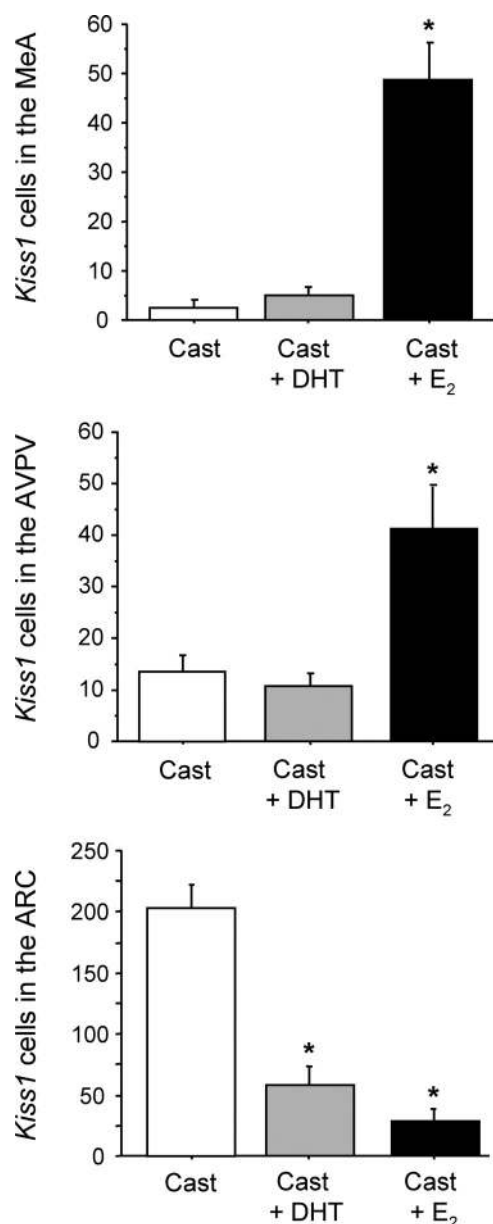


FIG. 8. Mean number of *Kiss1* cells in the MeA, AVPV/PeN, and ARC of mice that were GDX and treated with DHT, E₂, or nothing. There were significantly more *Kiss1* cells in the MeA of E₂-treated mice compared with either DHT-treated or untreated GDX mice. *, Significantly different ($P < 0.05$) from untreated, GDX control. Cast, Castrated.

direction of the gender bias differs between the two regions: in the AVPV/PeN, diestrous females have more *Kiss1* neurons than males (37, 42, 43), whereas in the MeA, males have more *Kiss1*-expressing neurons than diestrous females. Whether MeA *Kiss1* expression is also sexually dimorphic between intact males and proestrous females remains to be determined. Previous studies have found a similar male-biased sex difference in other aspects of the amygdala, including the MeA. For example, in the rodent MeA, the expression of vasopressin, cholecystokinin, and substance P are all greater in intact males than

intact females (48–50). Moreover, the overall size of the MeA region is larger in male rats than female rats (35). Thus, *Kiss1* gene expression follows the general pattern of male bias that appears to predominate in the MeA. However, at present, the functional significance of such a male-biased sex difference in MeA *Kiss1* levels is unknown.

Although we found that *Kiss1* expression is sexually dimorphic in both the AVPV/PeN and MeA of intact rodents, the mechanisms generating these *Kiss1* sex differences appear to differ between the two regions. In the AVPV/PeN, we previously determined that circulating sex steroid levels in adulthood are not responsible for the sex difference in the number of *Kiss1* neurons, because females have many more *Kiss1* cells in this region than males, even when adult males are treated with E₂ to simulate levels found in females (37). Rather, it appears that the sex difference in *Kiss1* expression in the AVPV/PeN is permanently organized by the prevailing sex steroid milieu during the perinatal critical period (37, 51). In contrast, our present results show that adult male and female mice and rats had the same number of *Kiss1* neurons in the MeA when the circulating sex steroid milieu was equalized between sexes. These findings indicate that the sex difference in the MeA *Kiss1* population reflects activational effects of the prevailing sex steroid milieu in the adult animal instead of a permanent developmental organization induced during the perinatal critical period (as with the AVPV/PeN). This is in agreement with findings that the sex difference in the overall size of the MeA is due to activational effects of sex steroids because equilibration of the sex steroid environment in adulthood eliminates the sex difference in the size of the MeA (35), analogous to the situation with *Kiss1* in the MeA.

We and others have previously shown that *Kiss1* expression in the rodent AVPV/PeN and ARC is differentially regulated by sex steroids, with T and E₂ stimulating *Kiss1* in the AVPV/PeN and inhibiting *Kiss1* in the ARC (6, 52). We now report that the *Kiss1* gene in the MeA is also regulated by sex steroids. Specifically, using both ISH and qPCR, we show that sex steroid treatment significantly up-regulates MeA *Kiss1* expression in both sexes, whereas GDX males and females not treated with sex steroids have low levels of *Kiss1* in the MeA. Thus, sex steroid regulation of *Kiss1* expression in the MeA mirrors that of *Kiss1* in the AVPV/PeN, being stimulatory in both regions. In concordance with this observation, we found that *Kiss1* levels in the MeA of females varies across the estrous cycle, peaking at proestrus when gonadal sex steroids are maximal and being much lower in diestrus and estrus when E₂ levels are reduced. The ability of sex steroids to up-regulate *Kiss1* in the MeA is similar to several other neuropeptides in this nucleus (e.g. vasopressin and substance P

in the MeA are up-regulated by T or E₂ and, conversely, decreased by GDX) (reviewed in Ref. 14).

In both rats and mice, E₂ and T induced *Kiss1* expression in the MeA. Because T can act either via AR or ER (after aromatization to E₂), it was unclear which receptor pathways mediated the effect of T on *Kiss1* expression in the MeA. The MeA shows high expression of both AR and ER (13, 53, 54), suggesting that either receptor pathway could conceivably mediate T's stimulation of *Kiss1* in this region. To address this issue, we evaluated whether DHT, a nonaromatizable androgen that can act via AR but not via ER, could stimulate *Kiss1* expression in the MeA. Despite the known presence of AR in the MeA, we found that DHT had no inductive effect on *Kiss1* expression in this region (or in the AVPV/PeN), unlike E₂ treatment, which robustly increased *Kiss1* levels in both areas. In contrast, DHT significantly inhibited the expression of *Kiss1* in the ARC, as did E₂. These data suggest that T up-regulates *Kiss1* in the MeA and AVPV/PeN after first being aromatized to E₂ and then acting through ER-dependent pathways. This mechanism is corroborated by the fact that both aromatase and ERs are expressed in the MeA (13, 54–58). However, it remains to be determined whether the effects of E₂ on *Kiss1* expression occur directly in MeA *Kiss1* neurons or indirectly via afferent circuits. Current studies in our lab are pursuing this important question.

The function of *Kiss1* neurons in the MeA is unknown. The amygdala has been implicated in numerous physiological and behavioral processes, including those relating to fear and anxiety, arousal and reward, stress, and social behavior. However, given the known roles of kisspeptin and its receptor in governing puberty onset and fertility, kisspeptin cells in the MeA may also play important modulatory functions in some aspects of reproduction. Indeed, the amygdala, including the MeA, has been implicated in modulating several facets of reproductive physiology, including sexual behaviors, ejaculation, gonadotropin secretion, ovulation, and pubertal maturation. For example, Fos is induced in the MeA of rats during sexual behavior (29), and electrical stimulation of the MeA results in elevated LH secretion (31, 59). Moreover, in rodents, pheromones and olfactory cues regulate reproductive function, and the MeA is a primary relay station linking the olfactory systems with reproductive nuclei, including to the preoptic area, AVPV, and ARC. The prominent location of the MeA in this olfactory-hypothalamic circuit indicates that the MeA is poised to play a role in olfactory or pheromone-induced changes in reproductive function; however, many of the specific cellular and molecular players in the MeA that play a role in this process are poorly characterized. Whether the newly identified MeA *Kiss1* neurons are in fact involved in the olfactory pathway or

play a role in sexual behavior or gonadotropin secretion in rodents remains to be established.

In summary, we report that in addition to its previously recognized expression in the hypothalamus, *Kiss1* is also expressed in the amygdala, specifically, the MeA. We provide evidence in two rodent species that the expression of *Kiss1* in the MeA is robustly regulated by sex steroids in both sexes and that this hormonal regulation occurs through ER-dependent pathways. We also show that levels of *Kiss1* change as a function of the estrous cycle, peaking at proestrus, and implying that MeA *Kiss1* signaling may play a role in ovulation or sexual behavior. Collectively, these findings identify a previously unrecognized population of extrahypothalamic *Kiss1*-expressing neurons that may play an important role in sex-steroid-regulated physiology and/or behavior. Future studies assessing the actions of kisspeptin signaling in the brain should consider the possible involvement of amygdala *Kiss1* neurons.

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