### Title

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# Developmental GnRH signaling is not required for sexual differentiation of kisspeptin neurons but is needed for maximal *Kiss1* gene expression in adult females

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Kisspeptin, encoded by *Kiss1*, stimulates reproduction. In rodents, one *Kiss1* population resides in the hypothalamic AVPV/PeN region. AVPV/PeN *Kiss1* neurons are sexually-dimorphic (greater in females), yet the mechanisms regulating their development and sexual differentiation remain poorly understood. Neonatal estradiol ($E_2$) normally defeminizes AVPV/PeN kisspeptin neurons, but emerging evidence suggests that developmental $E_2$ may also influence feminization of kisspeptin, though exactly when in development this process occurs is unknown. Additionally, the obligatory role of GnRH signaling in governing sexual differentiation of *Kiss1* or other sexually-dimorphic traits remains untested. Here, we assessed whether AVPV/PeN *Kiss1* expression is permanently impaired in adult *hpg* (no GnRH or $E_2$) or C57BL6 mice under different $E_2$ removal or replacement paradigms. We determined that 1) despite lacking GnRH signaling in development, marked sexual differentiation of *Kiss1* still occurs in *hpg* mice; 2) adult *hpg* females, who lack lifetime GnRH and $E_2$ exposure, have reduced AVPV/PeN *Kiss1* expression compared to WT females, even after chronic adulthood $E_2$ treatment; 3) $E_2$ exposure to *hpg* females during the pubertal period does not rescue their sub-maximal adult *Kiss1* levels; and 4) in C57BL6 females, removal of ovarian $E_2$ before the pubertal or juvenile periods does not impair feminization and maximal adult AVPV/PeN *Kiss1* expression nor the ability to generate LH surges, indicating that puberty is not a "critical period" for *Kiss1* development. Thus, sexual differentiation still occurs without GnRH, but GnRH or downstream $E_2$ signaling is needed sometime before juvenile development for complete feminization and maximal *Kiss1* expression in adult females.

The neuropeptide kisspeptin, encoded by *Kiss1*, is critical for puberty and fertility (1, 2). In rodents, kisspeptin-synthesizing neurons reside in the hypothalamic arcuate nucleus and in the hypothalamic continuum encompassing the anterior ventral periventricular nucleus and neighboring rostral periventricular nucleus (AVPV/PeN) (3-5). In adulthood, estradiol ($E_2$) suppresses *Kiss1* levels in the arcuate but robustly elevates *Kiss1* expression in the AVPV/PeN (5-7). Because kisspeptin directly stimulates GnRH activation (8-10), and AVPV/PeN *Kiss1* neurons express ER$\alpha$ (7), these neurons likely mediate $E_2$'spositive feedback effects on GnRH/LH secretion (i.e., the female LH surge) (11). Supporting this, in female rodents, there is an evening increase in AVPV/PeN *Kiss1* neuronal activation that coincides with the circadian onset of GnRH neuron activation and the LH surge (12-14). Moreover, *Kiss1r* KO female mice, which lack kisspeptin-Kiss1r signaling, cannot produce an $E_2$-induced LH surge (15, 16).

Supporting a role in the sexually-dimorphic LH surge event, AVPV/PeN kisspeptin neurons are themselves sexually dimorphic in cell number and *Kiss1* mRNA expression (greater in females than males) (3, 5, 17). Although detectable *Kiss1* and kisspeptin expression in the rodent

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Abbreviations:

AVPV/PeN does not first emerge until juvenile life (3, 18-20), we and others have demonstrated that sexual differentiation of AVPV/PeN *Kiss1* neurons is permanently induced via developmental sex steroid signaling in the neonatal period (i.e., an organizational effect of neonatal steroids) (5, 17, 21). Neonatal female rodents given T or $E_2$ at or shortly after birth have reduced, male-like numbers of AVPV/PeN *Kiss1* neurons later in adulthood (5, 17, 22), regardless of adult sex steroid levels. Conversely, removing sex steroids via castration from neonatal males has the opposite effect, producing higher female-like *Kiss1* expression in the AVPV/PeN in adulthood (17). The precise mechanisms by which perinatal $E_2$ directs the development and sexual differentiation of AVPV/PeN *Kiss1* neurons are still unknown, but may include epigenetic modifications of *Kiss1* expression (23).

Organizational sex differences can only be induced during specific developmental ages or "critical periods", outside of which sexual differentiation cannot be altered. For example, sex steroid treatment at birth permanently masculinizes the POA volume in rats, whereas T given later in juvenile life no longer masculinizes this region (24, 25). Although the presence of a neonatal critical period is well-supported, it is currently unresolved whether puberty also represents another "critical period" for kisspeptin sexual differentiation. Several studies, primarily in hamsters, suggest that the pubertal phase represents a critical period for some sexually dimorphic traits (26-29). Moreover, Clarkson et al. (30) observed that gonadectomy in female mice before puberty (~PND 15) reduced AVPV/PeN kisspeptin levels in early adulthood, and that $E_2$ replacement initiated during puberty could rescue this deficit. It was therefore proposed that the developmental trajectory of kisspeptin neurons in the female AVPV/PeN is further feminized by $E_2$ acting in a pubertal "critical period". However, the Clarkson data are difficult to interpret because group differences in circulating $E_2$ at the time of brain collection were not controlled for ($E_2$ transiently upregulates adult kisspeptin via activational effects (7)). Therefore, contributions of potential organizing effects of $E_2$ during a critical pubertal period versus activational effects of $E_2$ at the time of sacrifice remain unsorted. Regardless, additional data from female aromatase KO mice and hypogonadal (*hpg*) mice (lacking GnRH and hence, gonadal $E_2$) suggest that developmental $E_2$ exposure may indeed influence maximal female-like kisspeptin development. Both aromatase KO and *hpg* adult females display reduced kisspeptin protein staining in the AVPV/PeN (assessed via immunohistochemistry), even after short-term $E_2$ replacement in adulthood (31, 32). These findings suggest that $E_2$ signaling is required at *some point* in development or adulthood for display of maximal female-like kisspeptin expression (i.e., complete feminization). However, exactly when $E_2$ is needed to complete kisspeptin feminization is unknown. Furthermore, because similarly-aged *hpg* males and females were not directly compared (31), it remains unclear if AVPV/PeN kisspeptin is still sexually dimorphic in *hpg* mice or, alternatively, if kisspeptin levels in *hpg* females are lowered all the way to male levels. Indeed, the role—if any—of GnRH signaling in directly or indirectly governing sexual differentiation remains a contentious issue. Intriguingly, *hpg* male mice were recently reported to display a normal neonatal testosterone surge at birth (33), suggesting that sexual differentiation might still occur despite absent GnRH; however, this was not assessed.

We sought to answer several unresolved issues regarding AVPV/PeN *Kiss1* sexual differentiation and development. We determined 1) if sexual differentiation of AVPV/PeN *Kiss1* is impaired in *hpg* mice (no GnRH signaling), such that adult *hpg* males and females have similar *Kiss1* levels, 2) if AVPV/PeN *Kiss1* mRNA expression is, like previously-reported kisspeptin protein levels, reduced in adult *hpg* females, thereby distinguishing whether the lower kisspeptin protein in *hpg* mice reflects impaired *Kiss1* gene expression or impaired translation, 3) if short-term or long-term $E_2$ exposure in adulthood can rescue reduced *Kiss1* levels in *hpg* females, 4) if pubertal $E_2$ exposure can permanently restore maximal *Kiss1* expression in adult *hpg* females, and 5) if removal of gonadal $E_2$ during the pubertal or juvenile periods in C57BL6 females permanently reduces AVPV/PeN *Kiss1* levels or LH surges in adulthood.

## Materials and Methods

### Animals

C57BL6 mice or hypogonadal (*hpg*) and wild-type (WT) littermates (Jackson Labs) were used. Adult *hpg* homozygous mice lack GnRH and are therefore infertile, have undeveloped gonads, and absent gonadal sex steroids. *Hpg* litters were genotyped after weaning (postnatal day [PND] 21) via PCR analysis of tail DNA. In all experiments, weaned mice were provided food and water ad libitum and housed 2-3 under a 12-12 light-dark cycle (lights off at 1800h). All experiments were approved by the local University Animal Care and Use Committee.

### Gonadectomies and Hormone Treatments

Either in adulthood or development, mice were anesthetized with isoflurane and bilaterally gonadectomized (GDX) through a single ventral midline incision. The abdominal musculature was sutured with sterile chromic gut and the skin incision closed with sterile wound clips (adults) or sterile suture (juvenile/prepubertal mice).

Because $E_2$ transiently upregulates AVPV/PeN *Kiss1* expres-

sion in adults via activational effects (7, 23), $E_2$ levels were equalized among groups prior to brain collection. Some adult GDX mice (see specific experiments) received a subcutaneous Silastic implant filled with 4 mm of 17$\beta$-$E_2$ diluted 1:5 with cholesterol. These $E_2$ implants produce high physiological blood $E_2$ levels and significantly upregulate *Kiss1* expression in the AVPV/PeN of adult mice (7, 34). In experiment 4, peripubertal female mice were GDX and temporarily given a smaller 2 mm Silastic implant containing $E_2$ diluted1:20 with cholesterol, which a pilot experiment determined produced $E_2$ levels in the adult proestrus range. These peripubertal $E_2$ implants were removed 8 d later, and the skin incision closed with a sterile wound clip.

Experiment 6 assessed the ability of females to generate an $E_2$-induced LH surge, using a validated surge paradigm in which adult GDX females were given a subcutaneous implant containing 0.65 $\mu$g 17$\beta$-$E_2$ dissolved in sesame oil, as previously described (13, 16). This $E_2$ treatment produces proestrus-like $E_2$ levels and induces robust evening LH surges 2 d later (13, 16, 35).

## Brain Collection and In Situ Hybridization (ISH)

Mice were anesthetized with isoflurane and brains collected and frozen on dry ice before storage at −80°C. Frozen brains were sectioned on a cryostat into five sets of 20 $\mu$m sections, thaw-mounted on Superfrost-plus slides, and stored at −80°C until assaying. Single-label in situ hybridization (ISH) for *Kiss1* mRNA was performed using a validated murine *Kiss1* riboprobe (4). Briefly, one set of slide-mounted sections encompassing the entire AVPV/PeN region was fixed in 4% paraformaldehyde, pretreated with acetic anhydride, rinsed in 2X SSC (sodium citrate, sodium chloride), delipidated in chloroform, dehydrated in ethanols, and air-dried. Radiolabeled ($P^{33}$) antisense *Kiss1* riboprobe (0.04 pmol/ml) was combined with 1/20 vol yeast tRNA, heat-denatured, added to hybridization buffer, and applied to each slide (100 $\mu$l/slide). Slides were cover-slipped and placed in a humidity chamber at 55°C for 18 h. Following hybridization, slides were washed in 4X SSC and then placed into RNAse [37 mg/ml RNAse A in 0.15M 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 2X SSC at room temperature, slides were washed in 0.1X SSC at 62°C, dehydrated in ethanols, and air-dried. Slides were dipped in Kodak NTB emulsion, air-dried, and stored at 4°C for 4-6 d (depending on the assay) before being developed and coverslipped.

For double label ISH of *ER*$\alpha$ in *Kiss1* neurons (experiment 3), the single-label protocol was used with slight modification. Briefly, radio-labeled ($^{33}$P) antisense *ER*$\alpha$ (0.04 pmol/ml) and digoxigenin (DIG)-labeled *Kiss1* (1:500) riboprobes were combined with tRNA, denatured, and dissolved together in hybridization buffer, and applied to each slide (100 $\mu$l/slide) before overnight hybridization at 55°C. After Day 2's 62°C washes, slides were incubated in 2X SSC with 0.05% Triton X-100 containing 3% normal sheep serum (NSS) for 1 h at room temperature and then incubated overnight with anti-DIG antibody conjugated to alkaline phosphatase [(Roche) diluted 1:500 in Buffer 1 containing 1% NSS and 0.3% Triton X-100]. Slides were then washed with Buffer 1 and incubated with Vector Red alkaline phosphatase substrate (Vector Labs) for 1 h at room temperature. Slides were then air-dried, dipped in emulsion, stored at 4°C, and developed 7 d later.

## Quantification of ISH data and Statistical Analyses

ISH slides were analyzed blind to treatment using an automated custom grain-counting software (Dr. Don Clifton, University of Washington) that counts the number of silver grain clusters representing cells, as well as the number of silver grains in each cell (a semiquantitative index of mRNA content per cell) (36). Cells were considered *Kiss1* positive when the number of silver grains in a cluster exceeded that of background by 3-fold. A relative measure of total mRNA in the AVPV/PeN region was calculated by multiplying total number of cells by the number of silver grains per cell. For double-label ISH, red fluorescent DIG (*Kiss1*) cells were identified under fluorescence microscopy and the grain-counting software used to quantify the number of silver grains (*ER*$\alpha$ mRNA) overlying each cell. Signal-to-background ratios for individual cells were calculated by the program, and a cell was considered double-labeled if its ratio was $> 3$. All data are expressed as the mean ± SEM for each group. Differences were analyzed by ANOVA, followed by post hoc comparisons via Fisher's (Protected) LSD test. Statistical significance was set at $P < .05$.

## experiment 1: Is Kiss1 expression in the AVPV/PeN still sexually differentiated in hpg mice?

We recently reported that, despite having no GnRH signaling (and hence, no downstream goandotropin signaling), neonatal *hpg* male mice still display normal testosterone secretion at birth (33). Because neonatal testosterone secretion drives sexual differentiation, our prior finding suggested that sexual differentiation may occur normally in *hpg* mice, but this was not studied. This experiment directly tested whether AVPV/PeN *Kiss1* sex differences were still present in adult *hpg* mice. WT and *hpg* males and females were GDX on PND 38 and subcutaneously implanted with $E_2$ to control for activational effects of sex steroids. One week later (PND 45), mice were sacrificed and their brains collected (n = 5-7/group). Single-label ISH for *Kiss1* in the AVPV/PeN was performed and the number of *Kiss1*-expressing cells, the relative level of *Kiss1* mRNA per cell, and the relative amount of total *Kiss1* mRNA were measured.

## experiment 2: Does long-term $E_2$ exposure in adulthood rescue the submaximal Kiss1 expression in hpg females?

Experiment 1 showed that, despite still being sexually differentiated, adult *hpg* females have fewer detectable *Kiss1* neurons relative to WT females, even after short-term (1 wk) $E_2$ exposure in adulthood. However, since *hpg* females have never been exposed to $E_2$ during the juvenile, pubertal, or early adult periods, it may be that *hpg* females merely require a longer $E_2$ exposure than just 1 wk to complete the development and feminization of their *Kiss1* system. This experiment therefore tested if a chronic, longer-term exposure to $E_2$ could rescue the impaired *Kiss1* expression in adult *hpg* females and restore their *Kiss1* levels to those of WT females. First, to confirm our experiment 1 finding and also assess whether *Kiss1* is in fact $E_2$-responsive in *hpg* mice (contrary to a previous report looking at kisspeptin-ir levels (31)), we examined adult *hpg* and WT females that were GDX on PND 38 and given either an $E_2$ implant (n = 7-8/group) or nothing (n = 4-7/group); brains from all mice were collected one week later (PND 45) and analyzed for *Kiss1* in the AVPV/PeN using single-label ISH, as in experiment 1. Next, a separate cohort of

*hpg* and WT females were GDX on PND 38 and chronically implanted with $E_2$ (n = 8-9/genotype); a control group of PND 38 WT females was similarly GDX but not given any $E_2$ implant (n = 5). All mice were then sacrificed 22 d later (PND 60) and their brains collected. To prevent exhaustion of the $E_2$ implants, they were removed after 11 d and replaced with fresh implants for the remaining 11 d. Brains were analyzed for *Kiss1* mRNA levels in the AVPV/PeN using ISH.

### experiment 3: Do AVPV/PeN Kiss1 neurons of hpg females express less ERα than WT females?

Experiment 2 determined that although the *Kiss1* gene is $E_2$-responsive, AVPV/PeN *Kiss1* levels in *hpg* females remain submaximal even after chronic long-term $E_2$ exposure in adulthood. We hypothesized that this reduced *Kiss1* levels in hpg females may reflect a lower degree of ERα coexpression in *Kiss1* neurons of *hpg* than WT females, thereby producing lower *Kiss1* upregulation in the former genotype. To test this, we performed double-label ISH to assess and quantify the degree of ERα expression in AVPV/PeN *Kiss1* neurons in adult *hpg* and WT females (n = 4/genotype). The percent of AVPV/PeN *Kiss1* neurons expressing *ERα* and the relative amount of *ERα* mRNA per *Kiss1* cell were determined for each genotype.

### experiment 4: Does $E_2$ exposure during the pubertal period rescue the incomplete feminization of Kiss1 expression in hpg females?

Experiments 2 and 3 determined that although the *Kiss1* gene is $E_2$-responsive and expresses normal levels of ERα, *Kiss1* levels in *hpg* females remain submaximal even after chronic long-term $E_2$ exposure in adulthood. This suggests a developmental problem rather than lack of adult sex steroid exposure. Previous rodent studies suggested that the pubertal period is a "critical period" for sexual differentiation of some brain traits, and this may possibly also be true for AVPV/PeN kisspeptin. This experiment tested whether the lower *Kiss1* levels in adult *hpg* females reflects an absence of developmental $E_2$ exposure during the pubertal period. In our mouse colony, female puberty (reflected by vaginal opening) typically occurs between PND 26 and PND 29. WT and *hpg* females were therefore GDX just before puberty, on PND 22, and simultaneously implanted with $E_2$. The $E_2$ implant was left in for 8 d to span the pubertal period and then removed, after which all mice aged without additional $E_2$ exposure. At PND 56, females of both genotypes were implanted with either $E_2$ (to control for activational effects of adult $E_2$) or nothing and brains collected 1 wk later (PND 63) (n = 6-8/genotype). Single-label ISH for *Kiss1* in the AVPV/PeN was performed as in prior experiments.

### experiment 5: Is $E_2$ required during the juvenile and/or pubertal period for complete feminization of AVPV/PeN Kiss1 neurons?

Our previous experiments suggested that $E_2$ is needed sometime in development for complete feminization of the AVPV/PeN *Kiss1* system. Previous rodent studies suggested that puberty is a "critical period" for sexual differentiation of some traits, but this has not been definitively studied for AVPV/PeN kisspeptin. Here, we tested this possibility in normal C57BL6 females by removing gonadal $E_2$ before puberty and assessing the impact—if any—on *Kiss1* levels later in adulthood. We also assessed the necessity of $E_2$ exposure at even younger ages before puberty by testing whether permanent removal of gonadal $E_2$ before AVPV/PeN *Kiss1* expression first initiates in juvenile development disrupts feminization of this *Kiss1* system. C57BL6 females were GDX on either PND 14 (before the prepubertal and pubertal periods) or PND 9 (before the juvenile period and the first detectable AVPV/PeN *Kiss1* expression on PND 10 (18)). All GDX females were then aged to PND 35, when they were implanted with either $E_2$ (n = 8-9) or nothing (n = 5) and sacrificed 1 wk later (PND 42). Control C57BL6 females were left gonadally-intact throughout all of development, GDX and implanted with $E_2$ (n = 5-6) or nothing (n = 5-7) on PND 35, similarly sacrificed on PND 42. Brains were collected and *Kiss1* analyzed via ISH.

Because sexually-dimorphic AVPV/PeN kisspeptin is thought to drive the circadian-timed LH surge in females, a complementary experiment examined whether females lacking developmental $E_2$ could generate LH surges in adulthood. C57BL6 females were GDX on PND10 and aged in the absence of juvenile and pubertal $E_2$ exposure. On PND 54, they were implanted with an $E_2$ paradigm which normally induces evening LH surges 2 d later. Control females remained gonadally-intact throughout development, were GDX on PND 48, and similarly implanted with $E_2$ on PND 54. All mice were sacrificed on PND 56, either in the AM (when surges do not occur) or PM (at lights off, when surges normally occur) (n = 5-6/group). Blood was collected and serum LH levels determined using RIA (University of Virginia Ligand Assay Lab).

## Results

### experiment 1: Kiss1 expression in the AVPV/PeN is sexually dimorphic in hpg mice

This experiment tested if AVPV/PeN *Kiss1* expression is still sexually dimorphic or not in *hpg* mice (lacking GnRH signaling) with equalized adult $E_2$ levels. As expected, adult WT females had significantly higher *Kiss1* expression than WT males ($P < .01$ for *Kiss1* cell number, *Kiss1* mRNA per cell, and total *Kiss1* mRNA levels; Figures 1, 2). Interestingly, despite the lifetime absence of GnRH signaling, AVPV/PeN *Kiss1* was still sexually dimorphic in adult *hpg* mice, with *hpg* females having significantly higher *Kiss1* cell number (by 33%), *Kiss1* mRNA/cell (by 41%), and total *Kiss1* mRNA (by 87%) than *hpg* males ($P < .01$ for each measure; Figures 1, 2). However, feminization of *Kiss1* appeared submaximal in *hpg* mice: *Kiss1* cell number and total *Kiss1* mRNA levels were both approximately 40% lower in *hpg* females than WT females ($P < .01$; Figure 2). Likewise, defeminization (or masculinization) of *Kiss1* expression in *hpg* males was not maximal: for all measures, *Kiss1* levels were significantly higher ($P < .05$) in *hpg* males than WT males (25% to 86% higher, depending on the measure; Figure 2). Thus, while *Kiss1* expression is still sexually differentiated in *hpg* mice, there may be a developmental or adulthood

requirement for GnRH or downstream $E_2$ signaling for maximal *Kiss1* expression (perhaps denoting complete feminization) in females.

### experiment 2: AVPV/PeN Kiss1 expression is $E_2$-responsive in hpg females but still lower than in WTs, even after chronic adult $E_2$ exposure

Adult *hpg* and WT females were GDX on PND 38, treated for 1 wk with $E_2$ or vehicle, and their brains analyzed for AVPV/PeN *Kiss1* expression. As expected, WT GDX females had low *Kiss1* expression, whereas WT GDX+$E_2$ females had significantly higher *Kiss1* expression (*Kiss1* cell number, *Kiss1* mRNA per cell, and total *Kiss1* mRNA levels; $P < .01$ for each measure; Supplemental Figures 1, 2). Likewise, GDX+$E_2$ *hpg* females had significantly higher *Kiss1* cell number, *Kiss1* mRNA/cell, and total *Kiss1* mRNA than GDX *hpg* females without $E_2$ ($P < .01$ for each measure; Supplemental Figures 1, 2), indicating the *Kiss1* gene is still $E_2$ responsive. However, as in experiment 1, *Kiss1* levels were significantly lower in $E_2$-treated *hpg* females than $E_2$-treated WTs ($P < .05$; Supplemental Figure 2). Thus, while *Kiss1* expression is still responsive to $E_2$ in *hpg* females, the maximal level of *Kiss1* expression attained with short-term (1 wk) $E_2$ exposure is significantly lower (by 38%-45%, depending on the measure) in *hpg* than WT mice. We therefore next tested if longer-term exposure to $E_2$ in adulthood could rescue the submaximal *Kiss1* levels in *hpg* females. Adult WT females given chronic $E_2$ (3 wk) displayed the expected elevation in AVPV/PeN *Kiss1* levels relative to WT OVX without $E_2$ ($P < .01$; Figure 3). Likewise, adult *hpg* females given long-term $E_2$ for 3 wk had robustly elevated *Kiss1* levels, significantly greater than OVX females ($P < .01$; Figure 3). However, *Kiss1* levels in chronically $E_2$-treated *hpg* females were still significantly lower than in $E_2$-treated WT females, particularly for *Kiss1* cell number (25% lower) and total *Kiss1* mRNA levels (33% lower) ($P < .01$ for each, Figure 3). *Kiss1* mRNA per cell also showed a nonsignificant trend for lower levels in *hpg* females ($P = .10$).

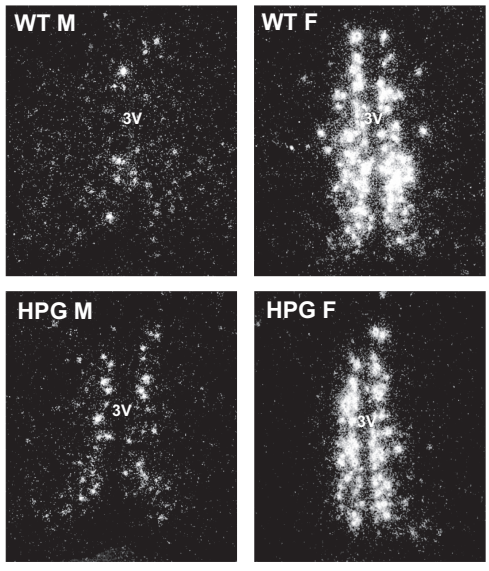


**Figure 1.** Representative photomicrographs of *Kiss1* gene expression, assessed via in situ hybridization, in the AVPV/PeN of adult *hpg* and WT male and female mice. All mice were treated with $E_2$ for 1 wk prior to brain collection. 3V = third ventricle.

### experiment 3: AVPV/PeN Kiss1 neurons of hpg females express normal ER$\alpha$ levels

Experiment 2 determined that AVPV/PeN *Kiss1* levels in *hpg* females remain submaximal even after long-term $E_2$

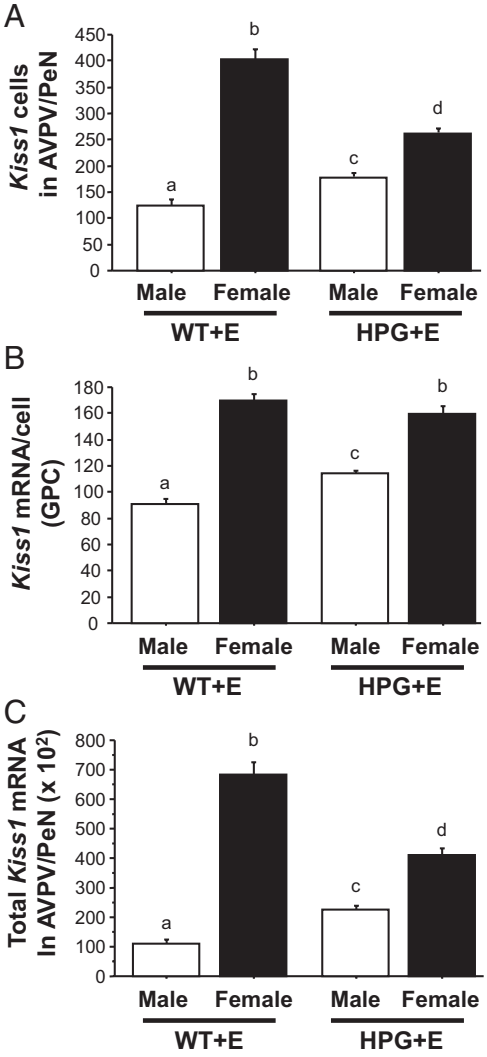


**Figure 2.** Sexually-dimorphic *Kiss1* expression in the AVPV/PeN of $E_2$-treatedadult (d45) *hpg* and WT males and females. **A)** Mean number of *Kiss1* cells in the AVPV/PeN. **B)** Mean relative levels of *Kiss1* mRNA per cell in the AVPV/PeN. **C)** Mean relative levels of total *Kiss1* mRNA in the entire AVPV/PeN region. For all measures, *Kiss1* was sexually dimorphic in *hpg* mice (higher in females). Bars labeled with different letters are significantly different from each other ($P < .05$).

exposure in adulthood. Here, we determined whether the reduced *Kiss1* expression in *hpg* females reflects lower ER$\alpha$ expression in their *Kiss1* neurons. We found that ER$\alpha$ was highly expressed in the AVPV/PeN of both *hpg* and WT females and present in most *Kiss1* cells in both genotypes (Figure 4A). Quantitatively, neither the percent of AVPV/PeN *Kiss1* neurons expressing *ER$\alpha$* nor the relative amount of *ER$\alpha$* mRNA per *Kiss1* cell differed significantly between genotypes (Figure 4B, C).

### experiment 4: Pubertal $E_2$ exposure is not sufficient to rescue the submaximal Kiss1 levels in hpg females

This experiment tested whether the lower *Kiss1* levels observed in adult *hpg* females reflects an absence of organizing $E_2$ exposure during the pubertal period, a potential "critical period". WT and *hpg* females were GDX on PND 22 and given $E_2$ for 8 d, and then left to age without additional $E_2$ exposure until PND 56. WT females given $E_2$ in adulthood had significantly higher *Kiss1* expression in all measures than WT females not given $E_2$ in adulthood ($P < .05$; Figure 5). A similar pattern was observed for *hpg* females with and without adulthood $E_2$ ($P < .05$; Figure 5). However, as in previous experiments, *hpg* females still had significantly lower *Kiss1* levels than WT females of the same treatment ($P < .05$; Figure 5), even though all mice were exposed to $E_2$ during the pubertal period. Thus, pubertal $E_2$ exposure is not sufficient to promote complete feminization of AVPV/PeN *Kiss1* neurons.

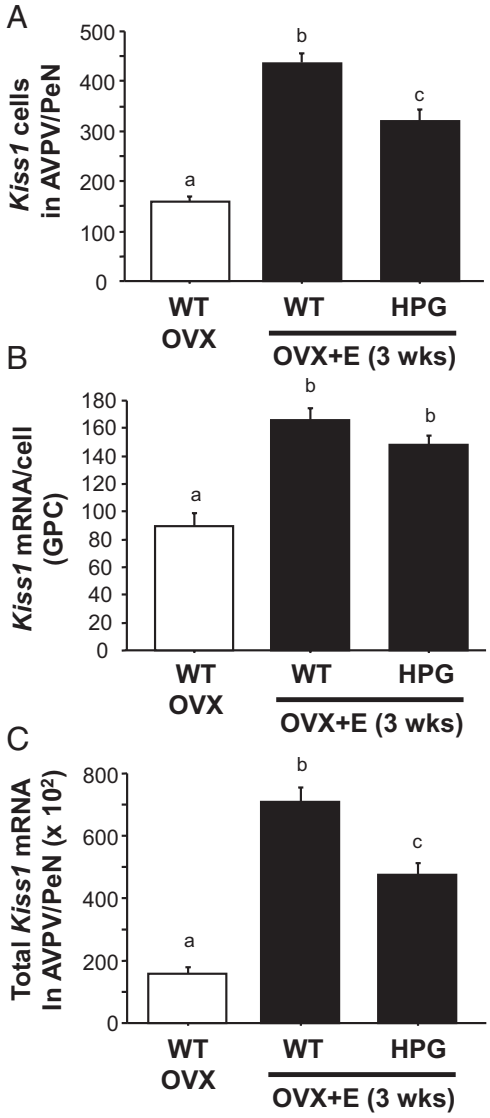


**Figure 3.** *Kiss1* expression in the AVPV/PeN of adult (d60) *hpg* and WT females with or without 22 d of chronic adulthood $E_2$ exposure. **A)** Mean number of *Kiss1* cells in the AVPV/PeN. **B)** Mean relative levels of *Kiss1* mRNA per cell in the AVPV/PeN. **C)** Mean relative levels of total *Kiss1* mRNA in the entire AVPV/PeN region. Bars labeled with different letters are significantly different from each other ($P < .05$).

### Experiments 5: $E_2$ is not required during the pubertal or juvenile periods for complete feminization of AVPV/PeN Kiss1 neurons

Here, we first tested in C57BL6 mice whether permanently removing $E_2$ exposure before the prepubertal and pubertal periods impacts the development and feminization of *Kiss1* in the AVPV/PeN. Control females left gonadally-intact throughout development and then GDX+$E_2$ on PND 35 displayed the expected high levels of *Kiss1* week later compared to control GDX females not given $E_2$ ($P < .01$; Figure 6). Similarly, females that were prepubertally GDX on PND14 and allowed to age without pubertal $E_2$ exposure demonstrated maximal *Kiss1* levels after $E_2$ treatment administered on PND 35 (Figure 6). There was no difference in any *Kiss1* measure between $E_2$-treatedfemales GDX before the prepubertal period (on PND 14) and control $E_2$-treatedfemales GDX on PND 35 (Figure 6), indicating that $E_2$ is *not* required during puberty for feminization and maximal *Kiss1* expression. Likewise, in the complementary experiment, C57BL6 females that were GDX on PND 9, before AVPV/PeN *Kiss1* expression first emerges in juvenile development, demonstrated maximal *Kiss1* levels after $E_2$ treatment later in young adulthood (given for 1 wk on PND 35; Figure 7). There was no significant difference in any *Kiss1* measure between females GDX before the juvenile period (on PND 9) or control females GDX on PND 35 (Figure 7), indicating that gonadal $E_2$ is *not* required during the juvenile period for complete feminization and maximal AVPV/PeN *Kiss1* expression.

Because sexually-dimorphic AVPV/PeN kisspeptin neurons govern the sexually-dimorphic LH surge, we also examined whether adult females lacking developmental $E_2$ could still generate normal LH surges. C57BL6 females GDX on PND 10 and given $E_2$ later on PND 54 displayed robust evening LH surges, similar to control females that were left gonadally-intact throughout development and

GDX on PND 48 (Figure 8). LH levels of both PM groups were significantly higher than AM control females ($P < .05$; Figure 8). Thus, $E_2$ is not required after PND 10 for complete sexual differentiation of the LH surge mechanism.

## Discussion

Sexual differentiation of AVPV/PeN *Kiss1* neurons may underlie several sexually-dimorphic processes, including the $E_2$-induced LH surge (positive feedback) and perhaps puberty. Although perinatal sex steroids, primarily $E_2$, permanently establish the sex difference in AVPV/PeN *Kiss1* expression, exactly *how* and *when* $E_2$ governs this process remains poorly understood. Here, we provide new evidence regarding the timing and necessity of GnRH and $E_2$ signaling in the sexual differentiation and development of AVPV/PeN *Kiss1* expression. We demonstrate that adult *hpg* females, who permanently lack GnRH (and hence, $E_2$), still have markedly higher AVPV/PeN *Kiss1* levels than *hpg* males, indicating that sexual differentiation does *not* require GnRH signaling. However, *Kiss1* gene expression in adult *hpg* females is markedly reduced compared to WT females, and these submaximal *Kiss1* levels cannot be rescued by short- or long-term adulthood $E_2$ treatment. This determination, in combination with our finding of normal *Kiss1*-ER$\alpha$ coexpression in adult *hpg* females, suggests a developmental rather than adulthood defect. We show that this developmental deficit is unlikely to be during the pubertal period, as *hpg* females exposed to $E_2$ during the pubertal stage still exhibit submaximal *Kiss1* levels in adulthood. Thus, puberty is not a critical period for *Kiss1* sexual differentiation, a finding confirmed by normal (complete) feminization of *Kiss1* in C57BL6 females after ovary removal on PND 9 or PND 14. Overall, our results indicate that while GnRH signaling is not needed for sexual differentiation of *Kiss1*, GnRH (likely via downstream $E_2$ secretion) is still needed sometime before juvenile development in females for complete feminization and maximal expression of AVPV/PeN *Kiss1* neurons.

Sexual differentiation of neural circuits, including AVPV/PeN *Kiss1*, is induced primarily by gonadal sex steroid secretion in perinatal males but not females. Because adult sex steroid secretion is dependent on upstream GnRH secretion, there is an assumption that perinatal androgen secretion (and hence, sexual differentiation) is also governed by GnRH signaling, but this has not been directly assessed. Our present results unquestionably demonstrate that AVPV/PeN *Kiss1* is still sexually dimorphic in the absence of GnRH signaling: adult *hpg* females had ~33%

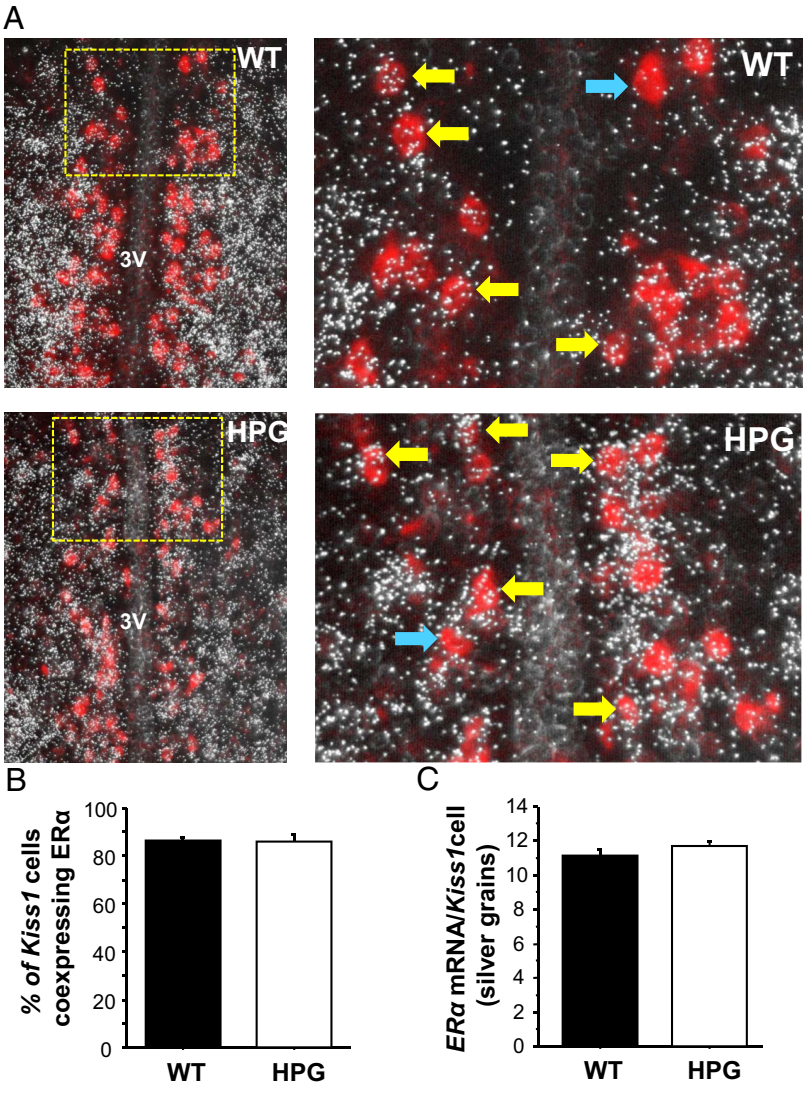


**Figure 4.** *ERα-Kiss1* coexpression in the AVPV/PeN of adult *hpg* and WT females. **A)** Representative photomicrographs of *ERα* expression in AVPV/PeN *Kiss1* neurons, assessed via double label in situ hybridization, in *hpg* and WT females. Yellow arrows designate examples of colabeled cells. Blue arrows designate example *Kiss1* cells lacking ERα. 3V, third ventricle. **B)** Mean number of *Kiss1* cells in the AVPV/PeN that coexpress ERα. **C)** Mean relative levels of *ERα* mRNA (silver grains) per *Kiss1* cell in the AVPV/PeN. There were no genotype differences in any measure.

more *Kiss1*-expressing cells and ~90% more *Kiss1* mRNA than *hpg* males. Therefore, GnRH signaling during the perinatal period (or any other developmental stage) is *not* required for sexual differentiation, at least for *Kiss1*. This conclusion is supported by our recent finding (33) that newborn *hpg* males, like WT males, produce a normal neonatal androgen surge, indicating that this androgen secretion—and by extension, sexual differentiation (as confirmed here)—is GnRH-independent.

Although sexual differentiation of AVPV/PeN *Kiss1* was clearly present in *hpg* mice, *Kiss1* mRNA expression in *hpg* females did not reach maximal WT female levels, perhaps reflecting incomplete feminization. Specifically, *Kiss1* cell number and total *Kiss1* mRNA were 38%-45% lower in *hpg* than WT females under controlled $E_2$ milieus. This impairment in female *hpg Kiss1* mRNA expression confirms and extends two recent reports of reduced AVPV/PeN kisspeptin protein in adult *hpg* and aromatase KO females (31, 32). Given the strong resemblance of the submaximal *Kiss1* phenotype in *hpg* females to that of aromatase KO females, the lower AVPV/PeN *Kiss1* levels in *hpg* females could reflect impaired organizational and/or activational effects of $E_2$ (owing to $E_2$'s dependenceon upstream GnRH signaling after the neonatal period). However, because neither short-term (1 wk) nor long-term (3 wk) $E_2$ treatment in adulthood fully restored *hpg* females' *Kiss1* levels to WT female levels (still ~33% lower in *hpg* females), we conclude that the submaximal

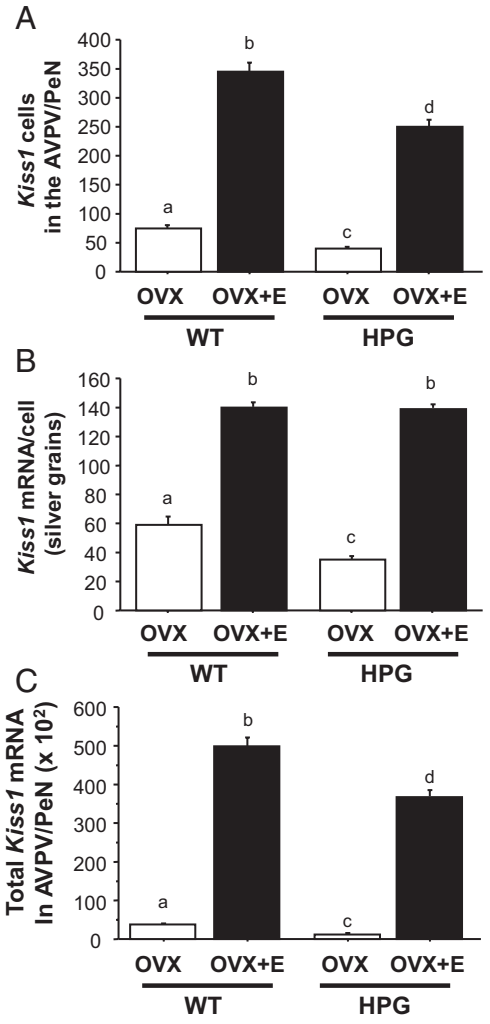


**Figure 5.** *Kiss1* expression in the AVPV/PeN of adult (d63) *hpg* and WT females after pubertal $E_2$ treatment from PND 22 to PND 30. **A)** Mean number of *Kiss1* cells in the AVPV/PeN. **B)** Mean relative levels of *Kiss1* mRNA per cell in the AVPV/PeN. **C)** Mean relative levels of total *Kiss1* mRNA in the entire AVPV/PeN region. Bars labeled with different letters are significantly different from each other ($P < .05$).

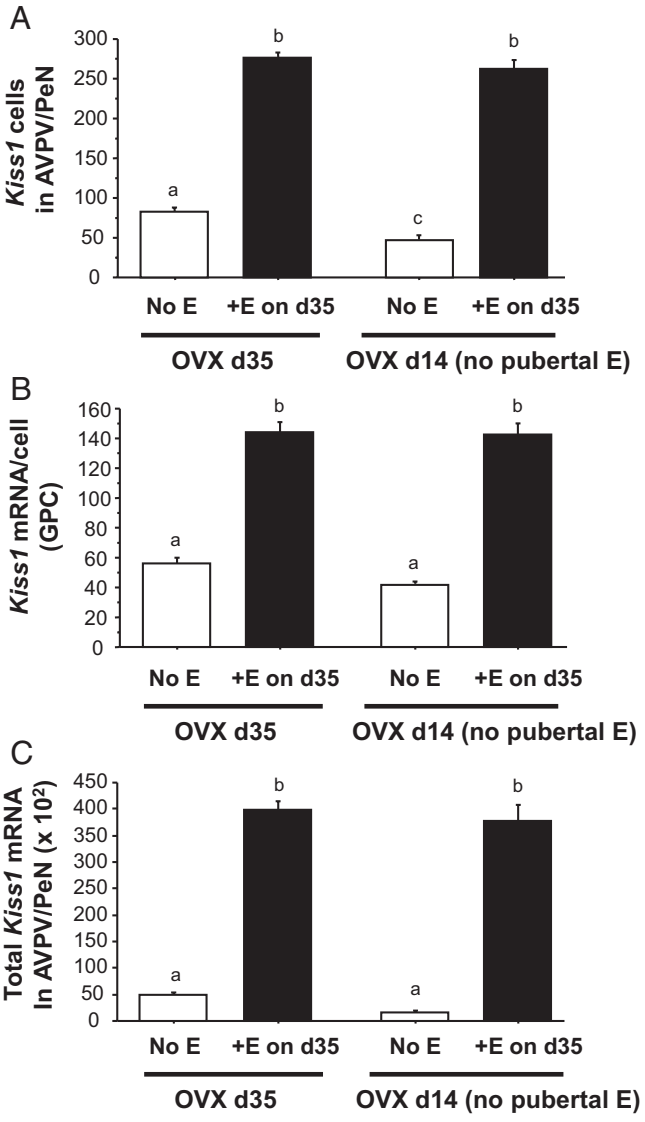


**Figure 6.** *Kiss1* expression in the AVPV/PeN of adult (d42) C57BL6 females that were GDX either after puberty, on PND 35, or before puberty, on PND 14. On PND 35, mice from each GDX group were given either an $E_2$ implant for 1 wk or no implant. **A)** Mean number of *Kiss1* cells in the AVPV/PeN. **B)** Mean relative levels of *Kiss1* mRNA per cell in the AVPV/PeN. **C)** Mean relative levels of total *Kiss1* mRNA in the entire AVPV/PeN region. Bars labeled with different letters are significantly different from each other ($P < .05$).

*Kiss1* expression in *hpg* females is not simply due to deficient activational $E_2$ signaling in adulthood. Nor is the reduced *Kiss1* phenotype due to diminished ER$\alpha$-*Kiss1* coexpression in *hpg* mice, as demonstrated in experiment 3. We therefore addressed whether maximal female *Kiss1* levels might rely on organizational $E_2$ signaling in pubertal development because a pubertal "critical period" for other traits exists in hamsters (26-29). In addition, because $E_2$ exposure earlier in the first week of postnatal life *defeminizes* (or masculinizes) *Kiss1*, any feminizing effect of $E_2$ on *Kiss1* would not be at perinatal development. We therefore first tried to rescue the submaximal *Kiss1* phenotype by exposing *hpg* females to $E_2$ during the pubertal period (PND 22-30). However, such pubertal $E_2$ treatment was not sufficient to restore *hpg* females' *Kiss1* levels to WT female levels, indicating that the pubertal period is unlikely to be a "critical period" for *Kiss1* sexual differentiation. This conclusion was supported by our findings that C57BL6 females ovariectomized before the prepubertal (PND 14) or juvenile (PND 9) periods and aged thereafter in the absence of gonadal $E_2$ were able to display maximal AVPV/PeN *Kiss1* levels later in adulthood. Thus, $E_2$ exposure during the juvenile or pubertal periods is not necessary for complete *Kiss1* feminization and maximal female *Kiss1* expression.

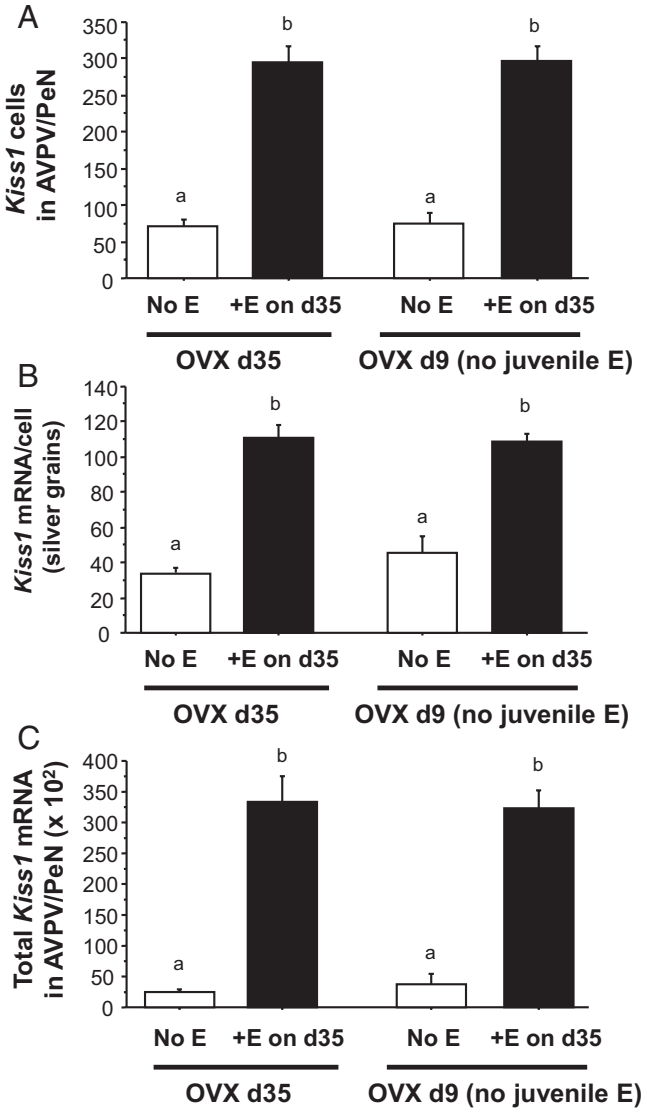


**Figure 7.** *Kiss1* expression in the AVPV/PeN of adult (d42) C57BL6 females that were GDX either after puberty, on PND 35, or before the juvenile period and time of first *Kiss1* expression, on PND 9. On PND 35, mice from each GDX group were given either an $E_2$ implant for 1 wk or no implant. **A)** Mean number of *Kiss1* cells in the AVPV/PeN. **B)** Mean relative levels of *Kiss1* mRNA per cell in the AVPV/PeN. **C)** Mean relative levels of total *Kiss1* mRNA in the entire AVPV/PeN region. Bars labeled with different letters are significantly different from each other ($P < .05$).

As discussed above, neither the juvenile nor pubertal periods appear to be "critical periods" for $E_2$'s ability to organize *Kiss1* feminization and enable maximal *Kiss1* expression in adulthood. Still, our findings of submaximal *Kiss1* expression in *hpg* females indicates that GnRH signaling, likely via downstream $E_2$ signaling, is required *at some point* in development for promoting maximal feminization of *Kiss1* neurons. Exactly when $E_2$ acts for such a process remains unknown, but our data suggest it is before PND 9. However, because $E_2$ exposure before birth (37) or soon after birth (5, 17) defeminizes (or masculinizes), rather than feminizes, *Kiss1* neurons, $E_2$ would seemingly have to act after this perinatal critical period to induce feminizing effects. Thus, $E_2$ might act between the perinatal critical period and PND 9 to completely feminize kisspeptin neurons, though this would be a very small temporal window and the ovaries are virtually quiescent in steroid production at this period. Unfortunately, ovariectomizing mice before PND 9 is technically challenging, making it difficult to study via this method. An alternate

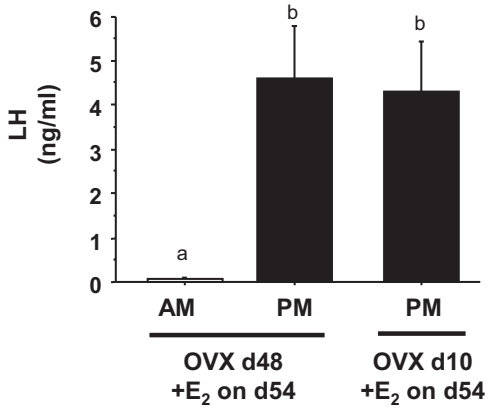


**Figure 8.** LH surge in mice with and without juvenile and pubertal $E_2$ exposure. C57BL6 females were GDX either before the juvenile period, on PND 10, or later in adulthood, on PND 48. All mice were given a positive feedback dosing of $E_2$ on PND 54 and LH was measured 2 d later in either the AM or PM. Bars labeled with different letters are significantly different from each other ($P < .05$).

possibility is that the developmental effects of $E_2$ are dose-dependent rather than age-dependent, with high neonatal $E_2$ permanently defeminizing/masculinizing *Kiss1* and lower (but not absent) neonatal $E_2$ enabling complete feminization. Again, this possibility is also difficult to assess, given that mouse neonatal $E_2$ levels are far too low to measured using conventional hormone assays. Lastly, brain-derived, locally-produced $E_2$ may possibly contribute to the feminization of kisspeptin neurons, though exactly how GnRH signaling (or its absence in *hpg* mice) would influence neural $E_2$ synthesis is unclear.

In conclusion, we show that AVPV/PeN *Kiss1* is still markedly sexually dimorphic in *hpg* mice, indicating that sexual differentiation occurs in the absence of GnRH signaling. However, despite normal ERa-*Kiss1* coexpression, *Kiss1* expression levels in *hpg* females are not maximal, even after chronic adulthood $E_2$ treatment, suggesting an organizational (developmental) rather than activational impairment. Because $E_2$ exposure to *hpg* females during the pubertal period does not rescue their lower *Kiss1* levels, puberty does not appear to be a critical period for *Kiss1* sexual differentiation in mice. Supporting this, gonadal $E_2$ removal prior to the pubertal or juvenile periods does not disrupt or prevent maximal *Kiss1* expression in adulthood. Overall, these findings demonstrate that sexual differentiation does not require GnRH signaling, suggesting that the neonatal endocrine events guiding sexual differentiation are GnRH-independent. Moreover, in addition to $E_2$'s well-described defeminizing effects on *Kiss1* at birth, developmental $E_2$ also acts sometime before juvenile life to promote complete feminization of *Kiss1* neurons, thereby enabling maximal *Kiss1* expression in females later in adulthood.

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