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Developmental sex differences in the peri-pubertal pattern of hypothalamic reproductive gene expression, including *Kiss1* and *Tac2*, may contribute to sex differences in puberty onset

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Abstract

The mechanisms regulating puberty still remain elusive, as do the underlying causes for sex differences in puberty onset (girls before boys) and pubertal disorders. Neuroendocrine puberty onset is signified by increased pulsatile GnRH secretion, yet how and when various upstream reproductive neural circuits change developmentally to govern this process is poorly understood. We previously reported day-by-day peri-pubertal increases (*Kiss1*, *Tac2*) or decreases (**Rfrp**) in hypothalamic gene expression of female mice, with several brain mRNA changes preceding external pubertal markers. However, similar pubertal measures in males were not previously reported. Here, to identify possible neural sex differences underlying sex differences in puberty onset, we analyzed peri-pubertal males and directly compared them with female littermates. *Kiss1* expression in male mice increased over the peri-pubertal period in both the AVPV and ARC nuclei but with lower levels than in females at several ages. Likewise, *Tac2* expression in the male ARC increased between juvenile and older peri-pubertal stages but with levels lower than females at most ages. By contrast, both DMN *Rfrp* expressionand *Rfrp* neuronal activation strongly decreased in males between juvenile and peri-pubertal stages, but with similar levels as females. Neither ARC KNDy neuronal activation nor *Kiss1r* expression in GnRH neurons differed between males and females or changed with age. These findings delineate several peri-pubertal changes in neural populations in developing males, with notable sex differences in kisspeptin and NKB neuron developmental patterns. Whether these peri-pubertal hypothalamic sex differences underlie sex differences in puberty onset deserves future investigation.

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CRediT authorship contribution statement

Sheila J. Semaan: Investigation, Data curation, Formal analysis, Visualization. **Alexander S. Kauffman:** Conceptualization, Supervision, Formal analysis, Visualization, Writing – original draft, Writing – review & editingReview, and Editing, Funding acquisition.

Declaration of competing interest

The authors have nothing to disclose.

Keywords

Puberty; Development; Sexual maturation; Reproduction; Kisspeptin; Kiss1; Kiss1r; Tac2; NKB; GnRH; RFRP-3; Rfrp; GnIH; Sex difference

1. Introduction

The neural and hormonal mechanisms regulating and timing puberty still remain elusive. Moreover, although it is commonly appreciated that puberty occurs earlier in girls than boys, the mechanistic reasons for this developmental sex difference are not entirely known. Likewise, the underlying causes of pubertal disorders, such as precocious or delayed puberty, are also not well known, nor are the reasons for sex differences in these disorders: precocious puberty is much more common in girls; delayed puberty is more prevalent in boys (Fechner, 2002; Ojeda et al., 2003; Ebling, 2005; Plant and Witchel, 2006; DiVall and Radovick, 2009; Lewis and Lee, 2009; Fenichel, 2012). To this end, pubertal studies encompassing detailed comparison of the two sexes are needed to gain insight into the neural and endocrine processes possibly controlling sex differences in normal pubertal onset and clinical pubertal disorders.

From a neuroendocrine perspective, puberty onset is defined as the developmental re-activation of the reproductive axis denoted by increased pulsatile release of GnRH from the brain and corresponding increased LH pulse secretion from the pituitary (Ojeda and Skinner, 2006; Plant and Witchel, 2006; Terasawa et al., 2013). Several afferent hypothalamic neural circuits have been implicated in governing GnRH pulse secretion, but exactly how these different upstream inputs change during peri-pubertal development to influence the onset of pubertal GnRH secretion still remains unknown (Kauffman, 2010; Tena-Sempere, 2012; Terasawa et al., 2013). Identified factors that directly or indirectly regulate GnRH neurons include stimulatory systems, like kisspeptin and neurokinin B (NKB), and inhibitory systems, such as dynorphin, RFRP-3, and GABA. In some cases, mutations in these signaling factors or their receptors alter or impair proper puberty onset or its completion. For example, puberty is dramatically impaired in humans, rats, or mice lacking functional kisspeptin (encoded by *Kiss1*) or its receptor (Kiss1r) (de Roux et al., 2003; Seminara et al., 2003; Lapatto et al., 2007; Topaloglu et al., 2012; Uenoyama et al., 2015), and humans with mutations in *Tac2*, encoding NKB, similarly have impaired puberty onset (Topaloglu et al., 2009; Young et al., 2010). In addition, animal experiments have demonstrated advanced induction of pubertal indices [e.g., LH secretion or vaginal opening (VO)] with chronic exogenous kisspeptin treatment, further implicating the kisspeptin system in sexual maturation (Navarro et al., 2004b; Shahab et al., 2005). Yet, it is still unclear exactly *when* endogenous kisspeptin or NKB participate in the pubertal process or whether these factors are under similar regulation between developing males and females. Indeed, developmental changes in brain reproductive gene expression, protein levels, and neuropeptide/neurotransmitter secretion are all likely important for influencing pubertal initiation and progression, and any of these may possibly contribute to known sex differences in puberty onset. However, the pubertal period stretches across many days and even weeks in rodents (months and years in larger species), requiring experimental

assessment at multiple ages within the pubertal transition to properly glean a complete picture of critical puberty changes. As reviewed previously (Semaan et al., 2013), in most rodent studies, developmental changes in reproductive brain populations (kisspeptin, NKB, RFRP-3, etc.) have typically been compared in one sex before and after puberty (or sometimes with a single pubertal age in between), with few analyses across *multiple* peri-pubertal ages and also directly between the sexes, Thus, little is known in rodents about the pattern of sequential changes in both sexes at multiple developmental stages leading up to or during the pubertal transition, especially in the ARC or DMN regions, and how this might differ between males and females. Moreover, while several prior studies in non-rodent species indicated moderate pubertal increases in ARC *Kiss1* and/or *Tac2* levels (Shahab et al., 2005; Redmond et al., 2011; Nestor et al., 2012; Li et al., 2020; Aerts et al., 2021), a few large-animal studies have detected minimal changes (Aerts et al., 2021; Harlow et al., 2021). In addition, the majority of non-rodent studies to date have been performed only in females and have not assessed the pubertal pattern in males or directly compared the male and female patterns to assess sex differences.

To gain temporal and spatial resolution of pubertal changes in rodent reproductive circuits, we previously determined the day-by-day developmental profile of *Kiss1* and *Tac2* expression in the ARC and AVPV, and *Rfrp* expression in the DMN, of female mice before and during the early-to-mid pubertal transition (Semaan and Kauffman, 2015). We initially focused on just females to permit a very large number of peri-pubertal ages to be directly compared, analyzing a total of 12 ages between the late juvenile age of postnatal day (PND) 15 and mid-pubertal PND 30 (mean VO occurred ~ PND 28). Supporting prior findings measuring AVPV kisspeptin-ir neuron numbers in developing mice (Clarkson and Herbison, 2006; Clarkson et al., 2009), we found that AVPV *Kiss1* mRNA levels in females markedly increased across this pubertal period, rising a week before VO, whereas the ARC *Kiss1* system showed smaller yet significant increases, reaching adult levels just before VO (Semaan and Kauffman, 2015). ARC *Tac2* levels also increased over the peri-pubertal period, with slightly greater magnitude than ARC *Kiss1* levels, whereas ARC *Kiss1* neuronal activation, assessed via *cfos* induction, did not show major increases over time but was notably higher, by ~4-fold, at all pubertal ages compared to the virtually absent AVPV *Kiss1* neuron activation at these ages (Semaan and Kauffman, 2015). In addition, both *Rfrp* expression and *Rfrp* neuronal activation in the DMN dramatically decreased in females between the juvenile and early pre-pubertal period, correlating negatively with peri-pubertal increases in *Tac2* and *Kiss1* (Semaan and Kauffman, 2015). Interestingly, all these changes in brain gene expression began before the external morphological puberty marker of VO was observed, indicating that “neuroendocrine puberty onset” initiates in the brain days before peripheral markers of “morphological puberty onset” are detectable.

Our prior study in female mice (Semaan and Kauffman, 2015) gave insight into key neural changes in the female puberty process but lacked any information on males, and by extension, possible sex differences in pubertal mechanisms. Fortunately, that female study also collected and simultaneously assayed brains from male littermates at several peri-pubertal ages, but the male data were not previously analyzed. Given the valuable opportunity to directly compare peri-pubertal brains of both sexes, the present report complements our prior female study by analyzing the peri-pubertal male data and comparing

it directly with that of their female littermates. Because all male brains were originally assayed together with the female brains, we can directly compare this 'new' male data to the original female data at several peri-pubertal stages. This allowed us to determine 1) the developmental pattern of reproductive gene expression or neuronal activation in males during the early peri-pubertal process, and 2) if there are notable sex differences in patterns of neural reproductive gene expression (*Kiss1*, *Tac2*, *Rfrp*, *Kiss1r*) or *Kiss1* or RFRP-3 neuron activation that might underly sex differences in puberty onset (females before males).

2. Materials and methods

2.1. Animals and ages

Male and female C57Bl6 mice were housed in a 12-12 light-dark cycle (lights off at 1800h). Food and water were available *ad libitum*. Litters containing 7–9 mice/litter were weaned on postnatal day (PND) 20, with day of birth designated as PND 1. Weaned mice were housed in groups of 2–3 of same-sex littermates. Vaginal opening (VO) and preputial separation (PPS), commonly-used morphological markers of puberty in female and male rodents, respectively, were assessed daily from weaning until the day of sacrifice. Mice in the present study were sacrificed at PND 15, 20, 24, or 28 (n = 5–8 mice/age/sex). To avoid confound of litter effects, each age group possessed males and females from at least 3 different breeder pairs. All mice were sacrificed within a similar time of day, between 1100h and 1300h; brains were collected fresh frozen on dry ice and stored at −80 °C until being processed for *in situ* hybridization. All animal studies were conducted with prior approval of the UCSD IACUC.

Data from the females were previously reported (Semaan and Kauffman, 2015); that original study examined females at 12 ages: PND 15 and every day between PND 20 and PND 30. All brain assays for that study also included brains of male littermates for several peri-pubertal ages (PND 15, 20, 24, 28), to permit assessment of possible peri-pubertal sex differences. Due to overall ISH assay size limitations, the total number of animals was restricted and therefore we could only examine males at 4 ages (PND 15, 20, 24, 28) rather than the 12 ages studied for females. In this present report, ISH data from PND15, 20, 24 and PND 28 females from the prior 2015 study is directly and statistically compared to the previously-unreported male data at the same ages (originally assayed together with the female tissue). No females exhibited VO on PND 15, 20, or 24 whereas all the PND 28 females analyzed here had exhibited VO. By contrast, none of the male mice yet displayed PPS at any of the 4 ages, highlighting the known sex difference in pubertal onset.

2.2. Single-label radiolabeled in situ hybridization

Frozen brains from both sexes were cut on a Leica cryostat into 5 alternating sets of 20 μm tissue sections that spanned from the rostral forebrain (mid-medial septal region) through the caudal hypothalamus. Tissue sections were thaw-mounted onto Superfrost plus slides and stored at −80 °C. Single-label radiolabeled ISH assays containing both male and female tissue were carried out using well-characterized P33 riboprobes for *Kiss1* (Gottsch et al., 2004; Kauffman et al., 2009), *Tac2* (Kauffman et al., 2009; Esparza et al., 2020a), and *Rfrp* (Poling et al., 2012, 2014) applied to slides at 0.05 pmol/ml. Depending on the specific

assay (*Kiss1, Tac2*, or *Rfrp*), 1 brain set of slide-mounted sections containing the entire rostral to caudal extent of the AVPV, ARC, or DMN from both males and females was assayed, as previously described (Kauffman et al., 2009; Semaan et al., 2010, 2012; Poling and Kauffman, 2012; Poling et al., 2014; Stephens et al., 2016). At the conclusion of each assay, slides were dipped in Kodak NTB emulsion, dried, and stored in the dark at 4 °C for 6–8 days (depending on the specific assay) before being developed and cover-slipped.

2.3. Double-label in situ hybridization

Double label ISH assays for *Kiss1r* + *Gnrh, cfos* + *Kiss1*, and *cfos* + *Rfrp* were performed using a combination of radiolabeled and digoxigenin (DIG) RNA probes. Depending on the specific assay, 1 set of slide-mounted sections (from both sexes) spanning either the late medial septum (MS) through the end of the OVLT (where most GnRH cell bodies reside) or the entire rostral to caudal extent of the ARC or DMN (for *Kiss1* or *Rfrp* neurons) was assayed using DIG-labeled mouse *Gnrh, Kiss1, Tac2*, or *Rfrp* riboprobes (1:500) and radio-labeled (^{33}P) *Kiss1r or cfos* riboprobes (0.05 pmol/ml), following our lab's previously described doublelabel protocol (Semaan et al., 2010; Poling and Kauffman, 2012; Dror et al., 2013; Di Giorgio et al., 2014; Poling et al., 2017). At the end of the assays, slides were dipped in NTB emulsion, dried, stored in the dark at 4 °C, and developed 7–10 days later.

2.4. ISH assay quantification and statistics

Tissue sections from all ISH assays were analyzed with an automated microscopy imaging processing software (GRAINS; Dr. Don Clifton, Univ. of Washington). Slides were coded so that the investigators were blind to the various age groups and sex during analyses. For single-label ISH assays, to determine cell number, the GRAINS software counted the number of tightly-packed silver grain clusters exceeding a background threshold by at least 3-fold. The software program also determined the number of silver grains within each individual cell cluster ("grains/cell"; a commonly-used semi-quantitative index of mRNA content per cell) (Chowen et al., 1990). For each brain region, a relative measure of total mRNA levels for each gene was calculated by multiplying the total cell number in that brain area by the relative amount of mRNA content per cell (Navarro et al., 2011; Kim et al., 2013; Semaan and Kauffman, 2015). For each gene, we therefore quantified total cell number, relative mRNA level per cell (grains/cell), and total mRNA levels in the brain region (AVPV, ARC, or DMN).

For the double-label ISH assays, red fluorescent DIG-containing cells (representing *GnRH, Kiss1*, or *Rfrp* cells) were identified under fluorescent microscopy and the GRAINS software then used to objectively quantify the number of P33-induced silver grains (representing *Kiss1r* or *cfos* mRNA) overlying each DIG cell under dark field microscopy. As in prior studies, signal-to-background ratios for individual DIG cells were calculated, and a DIG cell was considered double-labeled with the P33 riboprobe if its signal-to-background ratio was >3 (Navarro et al., 2011; Di Giorgio et al., 2014; Poling et al., 2017; Esparza et al., 2020b).

All data in the study are expressed as the mean ± SEM per group. The female data from the 4 peri-pubertal ages presented here were previously reported as part of a larger

dataset (Semaan and Kauffman, 2015) and are shown again for new statistical comparisons with the male data, in order to directly evaluate possible pubertal sex differences. Sex differences at each age were determined via planned comparisons using Student's t-test. To track developmental changes between PND 15 and 28, planned comparisons between the 4 peri-pubertal ages (PND 15, 20, 24, 28) within each sex were assessed via ANOVA with Fisher's LSD post-hoc analysis. In all cases, statistical significance was designated as $p < 0.05$.

3. Results

3.1. Sex differences in AVPV Kiss1 expression during the peri-pubertal period

As we previously reported, in the female AVPV, *Kiss1* neuron cell number increased markedly and consistently throughout the peri-pubertal ages examined, reaching adult levels by PND 28, coincident with VO (Fig. 1). The relative level of *Kiss1* mRNA/cell and total *Kiss1* mRNA levels in AVPV in females also increased robustly and significantly across the peri-pubertal period with a similar pattern (Fig. 1). By comparison, males had much lower AVPV *Kiss1* gene expression levels across the peri-pubertal ages studied. Specifically, developing males had fewer overall *Kiss1* neurons, less *Kiss1* mRNA per cell, and lower total *Kiss1* mRNA levels in the AVPV than females at every age examined ($p < 0.05$ for each age, Fig. 1). However, both sexes showed a similar overall magnitude of increase in total AVPV *Kiss1* expression from their starting levels at PND 15 to PND 28 (~5.0 fold increase both sexes), the primary difference being males' values were much lower to begin with, by >90%, on PND 15 and remained >90% lower by PND 28.

3.2. Sex differences in ARC Kiss1 expression during peri-pubertal development

We previously reported that the number of *Kiss1* neurons in the ARC of females was similar between PND 15 and PND 20 but increased slowly and moderately over the pubertal transition from PND 20 to the time of VO at PND 28, showing significant increases between PND 24 and PND 28, relative to PND 20 ($p < 0.05$; Fig. 2). In males, there was a similar pattern of slow increase in ARC *Kiss1* cell numbers across the age groups, with a significant increase first detected at PND 24. At PND 24, males had slightly higher ARC *Kiss1* cell numbers than females, by ~15% ($p < 0.05$; Fig. 2B). More dramatic was the difference between the sexes in *Kiss1* mRNA content per cell and total *Kiss1* mRNA in the ARC region. Females had a very high amount of *Kiss1* mRNA per cell at PND 15 that dropped notably by PND 20 and did not change significantly at ages after that. By contrast, males had much lower ARC *Kiss1* mRNA per cell, by ~30%, on PND 15 than females ($p < 0.05$; Fig. 2C). There was no developmental change in *Kiss1* mRNA per cell in the ARC of males at the ages examined and the two sexes did not differ on this measure at PND 20, 24, or 28. Total *Kiss1* mRNA in the ARC was also dramatically higher in females than males on PND 15, by nearly 40% ($p < 0.05$; Fig. 2D). Between PND 20 and PND 28, both males and females showed a slow, steady increase in total *Kiss1* mRNA in the ARC that reach significance by PND 28 for both sexes ($p < 0.05$ vs PND 15; Fig. 2D); however, unlike at PND 15, there were no sex differences in total *Kiss1* mRNA levels in the ARC at these older peri-pubertal ages.

3.3. Sex differences in Tac2 expression in the ARC during the peri-pubertal period

We analyzed peri-pubertal changes in ARC expression of *Tac2*, which encodes NKB and is co-expressed in most ARC *Kiss1* neurons. As we previously reported, there was a moderate gradual increase in *Tac2* cell number in the ARC of females from PND 15 to PND 28 ($p < 0.05$; Fig. 3B), with *Tac2* cell numbers in females reaching adult levels around PND 24, earlier than ARC *Kiss1* cell number. Likewise, total *Tac2* mRNA levels in the female ARC showed small but significant increases at PND 24 and PND 28 ages compared to PND 15 and 20 ($p < 0.05$; Fig. 3C and D). In males there was also an increase in *Tac2* cell numbers and *Tac2* mRNA levels from PND 15 to PND 28, with the increases first detected at PND 20 ($p < 0.05$ vs PND 15; Fig. 3B-D). Interestingly, despite fairly similar trajectories of ARC *Tac2* levels across peri-pubertal development, males had significantly lower *Tac2* levels than females. Indeed, for all three measures (*Tac2* cell numbers, *Tac2* mRNA per cell, and total *Tac2* mRNA in the ARC), females had significantly higher mean levels, by ~30–40%, than males at PND 15, PND 20, and PND 28 ($p < 0.05$ for each measure at each age; Fig. 3B-D), along with non-significantly higher mean values at PND 24.

3.4. Neuronal activation of KNDy neurons in peri-pubertal males and females

We examined the developmental pattern of neuronal activation and firing, as this could be different from peri-pubertal changes in gene expression. In females, we previously determined that neuronal activation of ARC *Kiss1/NKB/Dyn* (KNDy) neurons, measured via *cfos* mRNA co-expression, was much greater at any given peri-pubertal age between PND 15 and PND 30 than that observed in AVPV *Kiss1* neurons, the latter of which had virtually no detectable neuronal activation (<5%) at all these ages (Semaan and Kauffman, 2015). However, the degree of ARC KNDy neuronal activation in females did not fluctuate significantly during the peri-pubertal period, remaining around 15–20% at most ages (Fig. 4A, C). In males, a similar pattern emerged, with ARC KNDy neurons showing around 15–25% activation on different peri-pubertal ages (Fig. 4C). Despite a higher % of male KNDy neurons showing activation on PND 28 than PND 15, this age difference was not statistically significant. Moreover, at none of the 4 peri-pubertal ages examined was KNDy neuron activation statistically different between the sexes (Fig. 4C).

3.5. Kisspeptin receptor co-expression in GnRH cells in peripubertal males and females

Kisspeptin's activation of pubertal GnRH secretion may be influenced by its ability to signal to GnRH neurons through Kiss1r. We therefore measured the peri-pubertal pattern of *Kiss1r* mRNA expression in GnRH neurons. In females, the % of *Gnrh* neurons co-expressing *Kiss1r* was maximal on PND 15 and did not change notably across the peri-pubertal period, ranging between 85% and 92% co-expression on average for each day (Fig. 5). In addition, the relative amount of *Kiss1r* mRNA in GnRH neurons was not significantly different in females at any age between PND 15 and PND 28 (Fig. 5). In males, like females, the % of *Gnrh* neurons co-expressing *Kiss1r* was also at maximal levels on PND 15 and did not change at subsequent ages, ranging between 88% and 92% on average. There were no statistical differences between males and females in the % of *Gnrh/Kiss1r* colocalization or relative amount of *Kiss1r* mRNA content in *Gnrh* neurons at any of the 4 peri-pubertal ages examined.

3.6. Peri-pubertal decreases in Rfrp expression in both sexes

RFRP-3, synthesized in the DMN, has been proposed to provide inhibitory input onto the reproductive axis (Anderson et al., 2009; Ducret et al., 2009; Poling et al., 2014). We previously showed for peri-pubertal females that both the number of *Rfrp* neurons and total *Rfrp* mRNA levels in the DMN region were highly elevated on PND 15 and dropped significantly around PND 20 ($P < 0.05$ vs PND 15), with further step-wise decreases thereafter until stabilizing at lower levels around PND 28 ($p < 0.05$ vs PND 15; Fig. 6B,D). In males, a fairly similar developmental pattern was found: PND 15 males had significantly higher *Rfrp* cell counts and total *Rfrp* mRNA in the DMN than at PND 20 ($p < 0.05$ for both measures), and these *Rfrp* levels further decreased again by PND 24 ($p < 0.05$ for both measures vs PND 15) after which they stayed at the same low levels at PND 28 (Fig. 6). There were no significant changes in *Rfrp* mRNA content per cell across the different ages examined for either males or females. Comparing males with females, there were no significant sex differences in any *Rfrp* expression measure at any of the 4 peri-pubertal ages examined.

3.7. Neuronal activation of Rfrp neurons in peri-pubertal males and females

We also examined the pubertal profile of *Rfrp* neuronal activation that might possibly change independently of changes in *Rfrp* gene expression. In the DMN region, along with the robust peri-pubertal decreases in *Rfrp* gene expression discussed above, we detected large decreases in both sexes of *Rfrp* neuron activation status, reflected by *cfos* mRNA co-expression (Fig. 7). Specifically, as shown previously for females, males displayed a marked decreased in *Rfrp* neuronal activation between PND 15 and PND 20 ($p < 0.05$ for each sex; Fig. 7B and C), with no further developmental changes thereafter in the later peri-pubertal period. Directly comparing the sexes, males and females demonstrated similar levels of *Rfrp* neuronal activation at PND 15, 20, and 28, though males had slightly higher (1.3-fold) *Rfrp* activation levels at PND 24 ($p < 0.05$; Fig. 7C).

4. Discussion

A number of hypothalamic reproductive systems, including kisspeptin and NKB, have been implicated in governing the onset and timing of sexual maturation (de Roux et al., 2003; Seminara et al., 2003; Topaloglu et al., 2009, 2012; Young et al., 2010; Toro et al., 2018; Vazquez et al., 2018), but how these various factors change developmentally and in relation to one another still requires better understanding. Moreover, given known sex differences in both normal puberty onset (earlier in females) and human pubertal disorders (precocious puberty is more prevalent in females, delayed puberty more common in males), it is informative to identify commonalities and differences between male and female reproductive neurons during different stages of peri-pubertal development. Indeed, the mechanisms underlying different puberty onsets and progression tempos in males and females is still poorly understood. The present study leveraged data from multiple large-scale ISH assays that combined both males and females, but for which only females had been previously analyzed. We report here notable sex differences in the developmental patterns of gene expression for several key hypothalamic neuropeptides (summarized in Fig. 8), including *Kiss1* in the ARC and AVPV, and *Tac2* in the ARC, while some other factors,

like *Rfrp* expression in the DMN, showed dramatic peri-pubertal changes that were similar in males and females.

In humans and rodents alike, females generally initiate sexual maturation before males. In mice, the timing of puberty onset is often assessed indirectly via peripheral morphological measures of VO in females and PPS in males, and these can be affected by multiple factors, including litter size, metabolic status, and stress. In our lab, when these factors are controlled for, VO in female mice typically occurs between PND 27–30 whereas PPS in males generally occurs between PND 30–33, mirroring typical puberty sex differences in girls and boys (~10 vs 12 yrs old, respectively). VO and PPS are preceded by earlier neuroendocrine and endocrine changes, including increases in GnRH, LH, and sex steroid secretion, and VO and PPS therefore perhaps better represent mid-stage puberty rather than the actual *onset* of puberty. In the present study, all females in the oldest PND 28 group exhibited VO and had therefore already initiated puberty whereas no PND 28 males had yet demonstrated PPS. Whether puberty onset from a neuroendocrine perspective had already begun in PND 28 males is unknown but likely given 1) that there were already several observed changes in reproductive neural genes and 2) that it takes time for gonadal androgen synthesis and action to ultimately induce PPS which typically manifests ~PND 30–33. Given technical limitations on ISH assay size, we were unfortunately not able to include additional groups of males at older ages, and future studies would benefit from analyzing similar measures in males and females at older pubertal ages. Still, the present findings identify several genes that show significant changes in the peri-pubertal period of males as well as several notable peri-pubertal sex differences (e.g., *Tac2, Kiss1*) that might ultimately relate to sex differences in puberty timing.

Kiss1r or *Kiss1* mutations impair puberty (de Roux et al., 2003; Seminara et al., 2003; Topaloglu et al., 2012), and non-human primates show increased *in vivo* and *ex vivo* hypothalamic kisspeptin secretion at later stages of puberty compared to prepubertal animals (Keen et al., 2008; Guerriero et al., 2012). However, exactly when endogenous kisspeptin signaling in the brain first increases in developing monkeys—or any other species—remains undetermined and the neuroanatomical source (ARC or AVPV or both) of the increased peri-pubertal kisspeptin secretion also has not been definitively specified. To address that, our prior report in female mice studied the day-by-day changes of *Kiss1* expression across 12 peri-pubertal days in both the AVPV and ARC. That study found that *Kiss1* expression in the AVPV robustly increased between PND 15 and PND 30, with increases beginning at least a week before VO (~PND 28); a similar pattern of female AVPV *Kiss1* was shown in the present study examining just the 4 peri-pubertal ages at which males were studied and matches reported pubertal increases in AVPV kisspeptin protein levels, detected with IHC, in female mice (Clarkson and Herbison, 2006; Clarkson et al., 2009). Our present study further demonstrated that, like females, males also showed marked developmental increases in AVPV *Kiss1* levels between PND 15 and PND 28. However, the absolute levels of *Kiss1* were much lower in males than females at all peri-pubertal ages examined, being ~10-fold lower at PND 28. Indeed, the *Kiss1* levels of PND 28 males were still significantly lower than levels in younger PND 15 females, perhaps suggesting minimal function in males during the pubertal period. These findings mirror a report of similar sexually-dimorphic

increases in kisspeptin protein levels in the AVPV of pubertal male mice (Clarkson and Herbison, 2006).

Whether the developmental rises in AVPV *Kiss1* levels in either sex are part of the normal puberty-triggering mechanism or if the observed sex differences in AVPV *Kiss1* levels relate to sex differences in puberty onset remain unknown. In adulthood, AVPV kisspeptin neurons are implicated in driving the sexually-dimorphic LH surge that only occurs in females (Herbison, 2008; Khan and Kauffman, 2012; Mohr et al., 2021), which likely reflects the large female-biased sex difference observed in this kisspeptin population. However, the AVPV has yet to be implicated in puberty onset. It is possible that the large increases in peri-pubertal AVPV *Kiss1* levels in both sexes are a *response* to puberty onset rather than a cause, as this neural population is potently stimulated by sex steroids (Smith et al., 2005; Kauffman et al., 2007; Robertson et al., 2009), which themselves increase during puberty. Indeed, given the role of GnRH pulses rather than GnRH surges in triggering the *beginning* of puberty, AVPV kisspeptin neurons may not participate in puberty onset, but this has not yet been confirmed or functionally tested. Regardless, AVPV kisspeptin may play a role in the *late* stages of puberty, at least in females, as the first ovulatory event preceding first estrous near the end of puberty is likely to depend on an AVPV kisspeptin-mediated LH surge. Supporting this, a prior study (Mayer et al., 2010) found that selective knockout of ERα from all kisspeptin neurons advances pubertal *onset* (likely due to prematurely activated ARC kisspeptin neurons) but delays pubertal *completion* as indicated by impaired first estrous (likely due to failed activation of AVPV kisspeptin neurons to induce first ovulation). Unfortunately, our present study did not collect brains at the circadian time when the LH surge normally occurs in female mice (around the time of lights off); future studies could assess AVPV kisspeptin neuron measures in peri-pubertal females at a circadian time closer to lights off to see if AVPV *Kiss1* neuron activation shows pubertal increases as predicted; this assessment could also be combined with analyses of ARC tanycytes which have been shown to modulate GnRH neuron signaling during the time of the surge (King and Rubin, 1994, 1995; Prevot et al., 2010) and may possibly also change during puberty. By contrast, whether AVPV kisspeptin might also play a role in later stages of male puberty remains uncertain given the dramatically lower AVPV *Kiss1* levels present in males at all ages into adulthood and the fact that males don’t normally exhibit LH surges.

Limited data from other species are in agreement with a large increase in AVPV kisspeptin levels across the pubertal period, suggesting this may be a conserved developmental pattern. As in our present study in mice, prior reports in rats similarly found increases in AVPV kisspeptin neurons or *Kiss1* levels during puberty or between prepubertal and adult ages (Takase et al., 2009; Takumi et al., 2011). Like rodents, in developing female sheep, the number of *Kiss1*-expressing cells in the POA increases during the peri-pubertal period (Redmond et al., 2011), a finding supported by another study that detected virtually no POA kisspeptin cells in prepubertal sheep unlike high cell numbers observed in adult females (Nestor et al., 2012). Similar analyses of developing male sheep have not yet been reported. Although primates, including humans, also possess anterior hypothalamic/POA kisspeptin neurons (Smith et al., 2010; Watanabe et al., 2014; Vargas Trujillo et al., 2017; Rumpler et al., 2021), changes in this population have not yet been studied during puberty.

Contrasting the AVPV, ARC *Kiss1* expression in rodents is already high both *in utero* and at birth and is readily expressed at high levels throughout postnatal development (Cao and Patisaul, 2011; Poling and Kauffman, 2012; Kumar et al., 2014). Matching our prior findings analyzing 12 days across the pubertal period in female mice (Semaan and Kauffman, 2015), several studies reported small to moderate increases in ARC *Kiss1* levels in female rats around puberty (Takase et al., 2009; Takumi et al., 2011; Lomniczi et al., 2013) though another mouse study reported no differences in ARC *Kiss1* levels between juvenile and adult females, as determined with qPCR of MBH micropunches (Gill et al., 2010). Our present data further demonstrate that peri-pubertal males also show similar moderate increases in ARC *Kiss1* neuron numbers and overall ARC *Kiss1* levels, by ~20–25%, between PND 15 and PND 28. The present male data builds upon a prior report in male mice also showing increased ARC *Kiss1* levels (measured with qPCR) between PND 14 and adulthood (Molnar et al., 2016) and a study showing gradual increases in ARC *Kiss1* cell numbers across pubertal development in male rats (Takumi et al., 2011). The rise in ARC kisspeptin neuron numbers/levels may possibly reflect an increased neural drive to initiate puberty, perhaps around PND 24–28 in male mice, based on the present data.

Interestingly, as shown previously, both the relative *Kiss1* mRNA levels per cell and total ARC *Kiss1* levels of females were highest at the juvenile stage (PND 15) and subsequently decreased substantially by the early peri-pubertal stage (PND 20). This female-exclusive prepubertal decrease in ARC *Kiss1* levels between the 2nd and 3rd weeks of life has also been observed in pre-pubertal female rats (Cao and Patisaul, 2011; Takumi et al., 2011) and might reflect increasing negative feedback of rising ovarian sex steroids which are known to dampen ARC *Kiss1* gene expression (Smith et al., 2005); if so, it suggests that neuroendocrine puberty (which would precede gonadal sex steroid increases) may begin before PND 20 in female mice. It is interesting that male mice did not demonstrate any decrease in ARC *Kiss1* levels at any of the 4 peri-pubertal ages studied, in agreement with prior reports in developing male rats (Cao and Patisaul, 2011; Takumi et al., 2011). Rather, despite peri-pubertal increases in ARC *Kiss1* cell numbers, *Kiss1* mRNA levels per cell were unchanged in male mice between PND 15 and PND 28 and were ~30% lower in PND 15 males versus PND 15 females. Similar sex differences in infantile and juvenile ARC *Kiss1* levels, favoring females, have been reported in rats (Cao and Patisaul, 2011; Takumi et al., 2011; Cao et al., 2012). It is tempting to speculate that this stark juvenile/prepubertal ARC *Kiss1* sex difference contributes to, or reflects, earlier puberty initiation in females than males, but this requires further testing to confirm.

Prior findings in non-rodent mammalian species are, for the most part, in agreement with the moderate increases in ARC kisspeptin levels observed across the peri-pubertal period in the present study, suggesting this increase may be a conserved developmental pattern among mammals. In non-human primates, ARC *Kiss1* mRNA levels (measured via qPCR) increased with puberty in both agonadal males and intact females (Shahab et al., 2005). Unfortunately, these male and female monkeys were not from the same experiment and were not studied at the same developmental ages (or under the same gonadal status), so it currently remains unknown if there are sex differences in the pubertal pattern of ARC *Kiss1* in monkeys as were identified in the present study in mice. As in primates, in ovary-intact sheep, ARC kisspeptin cell numbers were found to be higher in postpubertal

than prepubertal females (Nestor et al., 2012), a finding mirrored by another recent report in female sheep assessing ARC *Kiss1* mRNA across the pubertal period (Li et al., 2020). Two additional studies in peri-pubertal female sheep that were OVX + E_2 reported only minor to no increases in *Kiss1* mRNA in the ARC (Redmond et al., 2011; Aerts et al., 2021), though the lack of more notable increases may be due to the exogenous E_2 clamping down ARC *Kiss1* gene expression, since estrogen is known to negatively regulate *Kiss1* in the ARC. Regardless, further regression analysis by one of those studies determined that the number of *Kiss1* cells in the middle-ARC showed a positive correlation with pubertal increases in LH pulse frequency (Redmond et al., 2011), supporting the findings from the two ovary-intact sheep studies. Finally, in female gilts, no increase was observed in *Kiss1* cells in the ARC during puberty, suggesting there may be species differences (Ieda et al., 2014; Harlow et al., 2021), though as one studied noted this does not rule out a pubertal change in ARC kisspeptin secretion in this species. Unfortunately, aside from the one aforementioned study in monkeys, little data exists at present for pubertal changes in male ARC kisspeptin neurons in non-rodent species.

ARC *Kiss1* cells also co-express *Tac2*, the gene for NKB, and *Pdyn*, the gene encoding dynorphin (Goodman et al., 2007; Navarro et al., 2009; Lehman et al., 2013), and are therefore often referred to as KNDy neurons (Cheng et al., 2010). NKB is thought to stimulate the reproductive axis via paracrine activation of other KNDy neurons (Wakabayashi et al., 2010; Qiu et al., 2016; Herbison, 2018), and mutations in NKB or its receptor impair normal puberty in humans (Topaloglu et al., 2009; Young et al., 2010). Our prior puberty study focusing on females documented a notable (~30%) increase in ARC *Tac2* cell number and total *Tac2* mRNA levels in the ARC across the peri-pubertal period, with significant increases first evident around PND 24, several days before VO (Semaan and Kauffman, 2015). In the present study, males similarly showed a peri-pubertal increase in ARC *Tac2* expression, but with several notable differences compared to females. First, the rise in *Tac2* expression relative to PND 15 was first evident in males around PND 20 but then showed no additional subsequent increases between PND 20 and PND 28. Even more striking was the clear sex difference between males and females in *Tac2* expression at most peri-pubertal ages examined: both *Tac2* cell numbers and relative *Tac2* mRNA expression levels were significantly higher in females than males at PND 15 and again at PND 24 and 28, contributing to total ARC *Tac2* levels being 40–50% higher in females than males. It is unclear if this sex difference persists at additional ages beyond PND 28, though we previously showed that adult males and females exhibit similar numbers of *Tac2* cells in the ARC (Kauffman et al., 2009). Given the link between NKB mutations and pubertal disorders in humans, we hypothesize that the higher *Tac2* levels in the ARC of juvenile and peri-pubertal females versus males contributes, in part, to the earlier puberty onset in females.

Few prior studies have studied pubertal changes of NKB or *Tac2* in detail. Gill and colleagues similarly reported pubertal increases in *Tac2* levels, measured via qPCR in MBH micopunches, in female mice (Gill et al., 2012). In female sheep, two studies reported no significant change in ARC NKB or *Tac3* cell numbers or *Tac3* mRNA levels per cell between prepubertal and postpubertal ages (Nestor et al., 2012; Li et al., 2020). Another study comparing female sheep that were OVX + E2 at different developmental and adult

ages similarly found no major pubertal increase in NKB protein or mRNA levels in the ARC (Aerts et al., 2021), and a recent study in female pigs similarly reported no pubertal changes in ARC NKB numbers (Harlow et al., 2021). These findings suggest that the pubertal rise in ARC NKB levels in rodents may not generalize to sheep or other ruminants. At present, we are not aware of similar NKB studies in pubertal males, or comparing pubertal males to females, in non-rodent species.

In rodents, sheep, and other animal models, KNDy neurons also coexpress dynorphin. Unfortunately, in the present study, we did not have additional brain sets remaining to also assay ARC *Pdyn* (dynorphin). Given the proposed role of dynorphin in sculpting KNDy neuron firing and LH pulses (Wakabayashi et al., 2010; Goodman et al., 2013, 2022; Qiu et al., 2016), future studies should ascertain how *Pdyn* levels change in each sex during the pubertal transition to provide a more in-depth picture of KNDy neuron development and function during this critical developmental period. Interestingly, the few studies that have studied ARC dynorphin levels in relation to pubertal development have found that either there is a notable pubertal increase in dynorphin neuron numbers and cellular Pdyn mRNA levels in the ARC, as shown in female sheep (Li et al., 2020; Aerts et al., 2021), or there is a moderate decrease in the number of *Pdyn*-expressing neurons between prepubertal and postpubertal females, as measured in female pigs (Harlow et al., 2021). The reason for the species differences is not yet known, and similar peripubertal analyses in males have not yet been reported.

Unlike the observed changes in *Tac2* and *Kiss1* levels in both sexes, ARC KNDy neuron activation did not change notably over the peripubertal period in males or females and did not demonstrate any significant sex differences at the 4 ages examined. This outcome was surprising given the important role of these neurons in GnRH pulses (Clarkson et al., 2017; Herbison, 2018; Han et al., 2019), but may reflect the fact that the periodic “pulsatile-like” activation of KNDy neurons would not necessarily be synchronized between individual animals. This could explain the variability observed in this measure in both sexes. It is also possible that time of day was a factor and that pubertal KNDy neurons show circadian increases in neuronal activation, as has been reported for pubertal LH secretion in rats (e.g., higher in the late afternoon than mid-day) (Ojeda and Skinner, 2006). If so, assessment of KNDy neuron activation at a different circadian time may better uncover pubertal changes or sex differences in this measure. Alternatively, a more direct measure of neuron activity may be better for studying this issue (Han et al., 2019). Regardless it should be noted that in our prior female study (Semaan and Kauffman, 2015), the degree of mean neuronal activation was several-fold higher in ARC *Kiss1* neurons compared to AVPV *Kiss1* neurons at all peri-pubertal ages studied (~15–20% vs 2–5% *cfos* co-expression, respectively). That comparison suggests that ARC *Kiss1* cells generally exhibit greater neuronal activation during the peri-pubertal period than *Kiss1* neurons in the AVPV. Whether this regional difference relates to the proposed participation of ARC, but not AVPV, kisspeptin neurons in governing GnRH pulse secretion (a hallmark of puberty onset) remains to be tested. In the present study, we did not report AVPV *Kiss1* neuronal activation in males because their very low AVPV *Kiss1* gene expression made it difficult to analyze sufficient numbers of fluorescent *Kiss1* cells to determine % *cfos* co-expression. However, the few AVPV *Kiss1*

neurons we did identify in males lacked *cfos* induction (not shown), similar to female AVPV *Kiss1* cells.

In rodents, kisspeptin doses in juveniles are less effective at increasing LH secretion and activating GnRH neuron electrical activity than in adults (Han et al., 2005; Castellano et al., 2006), suggesting that kisspeptin's ability to stimulate GnRH neurons is lower at ages before puberty. In monkeys and rats, overall *Kiss1r* expression levels in the hypothalamus are lower in juveniles than adults (Navarro et al., 2004a; Shahab et al., 2005), though these *Kiss1r* levels were measured in whole hypothalamic chunks that combined multiple brain regions and cell-types. In the present study, we used double ISH to focus specifically on *Kiss1r* expressed in GnRH neurons, as such *Kiss1r* expression was shown to be both sufficient and necessary for fertility in mice (Kirilov et al., 2013). In both developing female and male mice, *Kiss1r* levels in *Gnrh* neurons, as well as the percentage of *Gnrh* neurons expressing *Kiss1r*, are at very high levels at PND 15 and do not vary at any subsequent age during the peri-pubertal transition. There were also no identifiable sex differences in *Kiss1* levels in *Gnrh* neurons at any of the 4 peri-pubertal ages, though we cannot rule out sex or developmental differences at juvenile ages before PND 15 (Herbison et al., 2010). Our findings suggest that kisspeptin's ability to signal to its receptor on GnRH neurons is similar between the sexes and already at high levels long before the occurrence of morphological pubertal indices; thus, sex differences in puberty onset are unlikely to be due to differences in peri-pubertal Kiss1r levels in GnRH neurons. Of note, we primarily analyzed the late MS and entire OVLT portion of the GnRH population, which together contain the most GnRH soma in mice and show high *cfos* induction in prior studies of adult female rodents (Lee et al., 1992; Wu et al., 1992; Dror et al., 2013); whether there are pubertal or sex differences in Kiss1r in other more rostral (olfactory bulb) or caudal (mid-to-late POA) GnRH sub-populations is unknown but probably unlikely given that virtually every GnRH cell we identified in the present study expressed Kiss1r irrespective of age examined. Regardless, beyond Kiss1r, there may be pubertal changes in other factors within GnRH neurons that may play a role in functional activation of these neurons during puberty. For example, pubertal increases in glutamate receptor have been reported in GnRH neurons of female rats (Gore et al., 1996), perhaps underlying enhanced stimulatory input at puberty; whether there are sex differences in the developmental pattern of those glutamate receptor changes is not known. Likewise, other factors in GnRH neurons, like GABA receptors or intracellular components underlying electrical firing and peptide secretion, could be worth comparing between sexes at different pubertal stages.

Data from multiple species suggests that, prior to puberty, the brain helps to sustain an inactive reproductive status by inhibiting GnRH and LH release, independent from gonadal steroid negative feedback (Foster et al., 1972; Bass et al., 1979; Plant, 1980; Plant and Zorub, 1982; Jean-Faucher et al., 1985; Ebling, 2005; Ojeda and Skinner, 2006; Plant and Witchel, 2006; Kauffman et al., 2009; Terasawa et al., 2013), though the specific neural factor(s) providing a "brake" on the reproductive axis before puberty remain unknown. A prior report found that DMN neurons synthesizing RFRP-3, an inhibitor of reproductive hormone secretion, were more numerous in young juvenile than adult mice (Poling et al., 2012). We therefore determined whether *Rfrp* neurons exhibit peri-pubertal changes coincident with puberty onset. In females, *Rfrp* neuron number and total *Rfrp* mRNA

dropped markedly and consistently between PND 15 and PND 28, after which these measures held steady at adult levels. We found a nearly identical pattern in males in the present study: PND 15 males exhibited very high levels of *Rfrp* expression and neuron numbers, which then dropped significantly in a continued step-wise manner at PND 20, PND24, and PND 28. In males, like females, overall *Rfrp* levels were ~50% lower on PND 28 than on PND 15. However, there were no sex differences in *Rfrp* expression at any of the 4 peri-pubertal ages examined. Like *Rfrp* gene expression, *Rfrp* neuron activation exhibited similar peri-pubertal decreases, by ~40–45%, in females and males. However, unlike the continued decreases in *Rfrp* gene expression across ages, the large drop in *Rfrp* neuronal activation in both sexes only occurred between PND 15 and PND 20, with little further change at older peri-pubertal ages. Given the proposed role of RFRP-3 as an inhibitor of reproductive hormone secretion (Kriegsfeld et al., 2006; Anderson et al., 2009; Ducret et al., 2009), the decreases in *Rfrp* expression and neuronal activation may possibly represent a lessening of RFRP-3-mediated inhibition of the reproductive axis before puberty onset, though this has not been tested. In mice, RFRP-3 neurons project directly to a subset of both ARC kisspeptin neurons and GnRH neurons (Poling et al., 2013) and a small percent of these neurons express receptors for RFRP-3 (Poling et al., 2013). Still, a majority of GnRH and kisspeptin neurons do not express RFRP-3 receptors or receive axonal connections from RFRP-3 neurons, leaving it uncertain if there is a direct functional connection between these neural systems. Moreover, knockout mice lacking GPR147 (an RFRP-3 receptor) have normal puberty onset, suggesting RFRP-3 may not play a key role in puberty timing (Leon et al., 2014), though other RFRP-3 receptors were still intact, leaving the issue unresolved. At minimum, our present data suggest that sex differences in puberty onset are unlikely due to RFRP-3 neurons, which showed identical developmental patterns between males and females.

5. Summary and conclusions

In summary, this study identified a number of significant changes in reproductive gene expression and neuron activation status across the peri-pubertal period in male mice, as well as several sex differences in these measures between peri-pubertal males and females (summarized in Fig. 8). The most notable changes in males between PND 15 and PND 28 were small to moderate increases in AVPV *Kiss1*, ARC *Kiss1*, and ARC *Tac2* expression, and more sizable reductions in *Rfrp* expression and *Rfrp* neuronal activation. It remains to be determined whether any of these observed changes actually reflect a key component in the pubertal timing mechanism versus other important developmental or physiological processes, or secondary responses to other changes (like increased sex steroid secretion) occurring during puberty. We hypothesize that the *Kiss1* and *Tac2* increases in both sexes relate to amplified stimulation of GnRH neurons and the maturing reproductive axis, whereas the decreases in *Rfrp* levels and neuronal activation may reflect direct or indirect disinhibition of neural reproductive circuits (kisspeptin or GnRH neurons) thereby helping to facilitate puberty onset, though this latter possibility is more tenuous than the more likely involvement of kisspeptin and NKB neurons. As with females and VO status, all the observed neural changes in males occurred well before any observance of PPS, the common morphological marker of male puberty. This further supports the notion that neuroendocrine

pubertal changes in the brain occur earlier than VO and PPS, suggesting that VO and PPS are not exact indicators of puberty *onset* but rather of pubertal *progression*. Finally, our findings identify several notable sex differences in the brains of developing males and females during the juvenile and peri-pubertal periods. Key among these were higher *Tac2* levels in females at most ages examined, greater ARC *Kiss1* levels in juvenile (PND 15) females than males, and robustly higher *Kiss1* levels in the AVPV at all ages. These findings build upon and extend prior studies in multiple species showing pubertal increases in *Kiss1* and *Tac2* levels, though to date more studies have been performed in females. Indeed, a current gap in knowledge is the minimal information on ARC *Kiss1* and *Tac2* changes during puberty in male non-rodent species, and whether sex differences exist in any of these measures as occurs in pubertal mice.

Given the proposed role of ARC KNDy neurons in causing GnRH pulses, we hypothesize that the female-favoring peri-pubertal ARC *Kiss1* and *Tac2* sex differences contribute, in part, to earlier puberty onset in females than males. Conversely, it is currently less clear if the peripubertal AVPV *Kiss1* sex difference plays a role in timing puberty onset or if the higher AVPV *Kiss1* levels in females simply reflect sex differences in LH surge generation demonstrated in adulthood.

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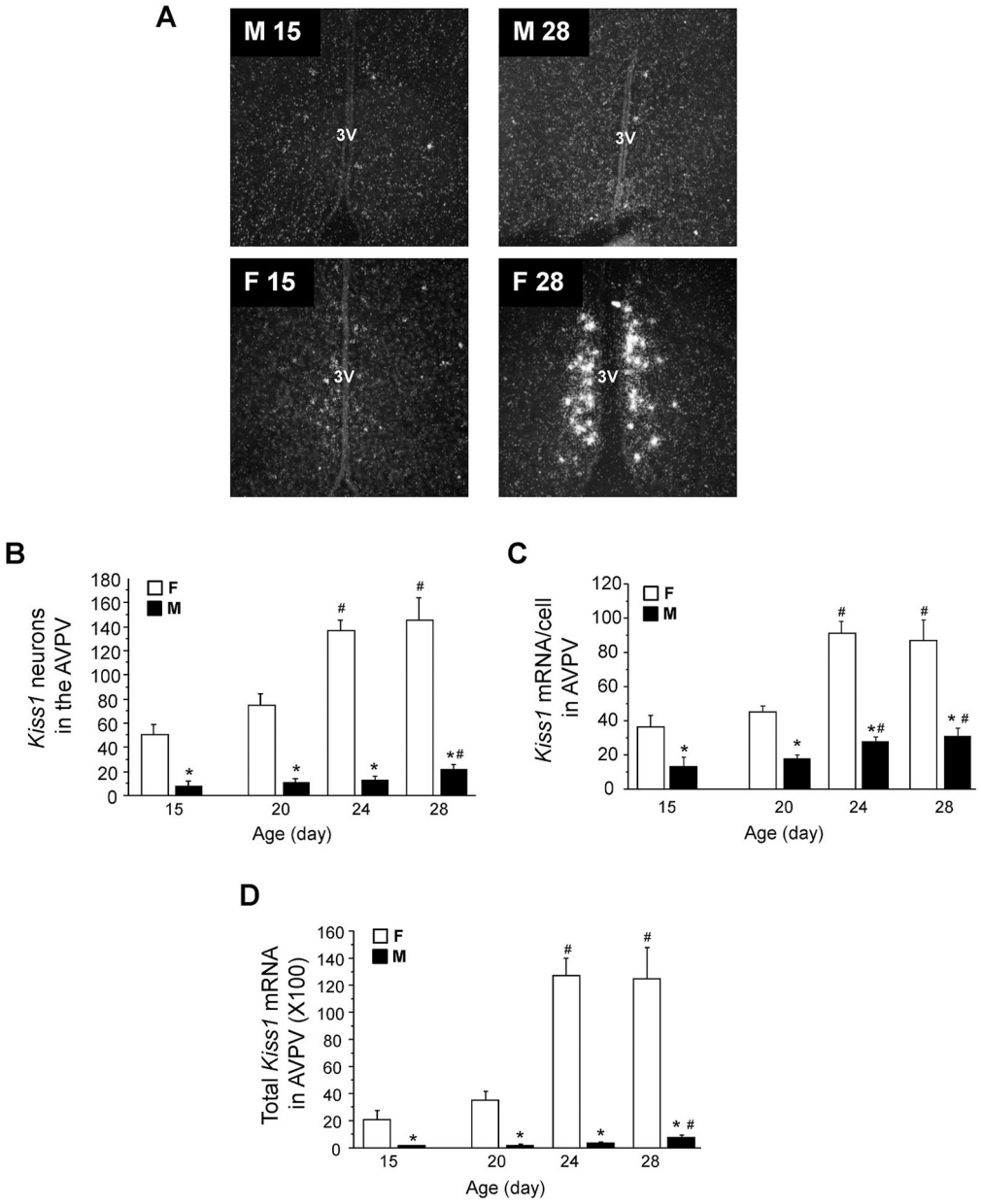

Fig. 1.

Kiss1 expression in the AVPV of male and female peri-pubertal mice. **A)** Representative images of *Kiss1* expression, determined by radio-labeled ISH, in the AVPV. 3V, third ventricle. **B)** Mean numbers of *Kiss1* neurons in the AVPV, **C)** mean *Kiss1* mRNA content per neuron in the AVPV, and **D)** mean relative total *Kiss1* mRNA in the AVPV of male and female mice between PND 15 and PND 28. #, $p < 0.05$ vs PND 15 of the same sex. *, $p < 0.05$ vs females of the same age.

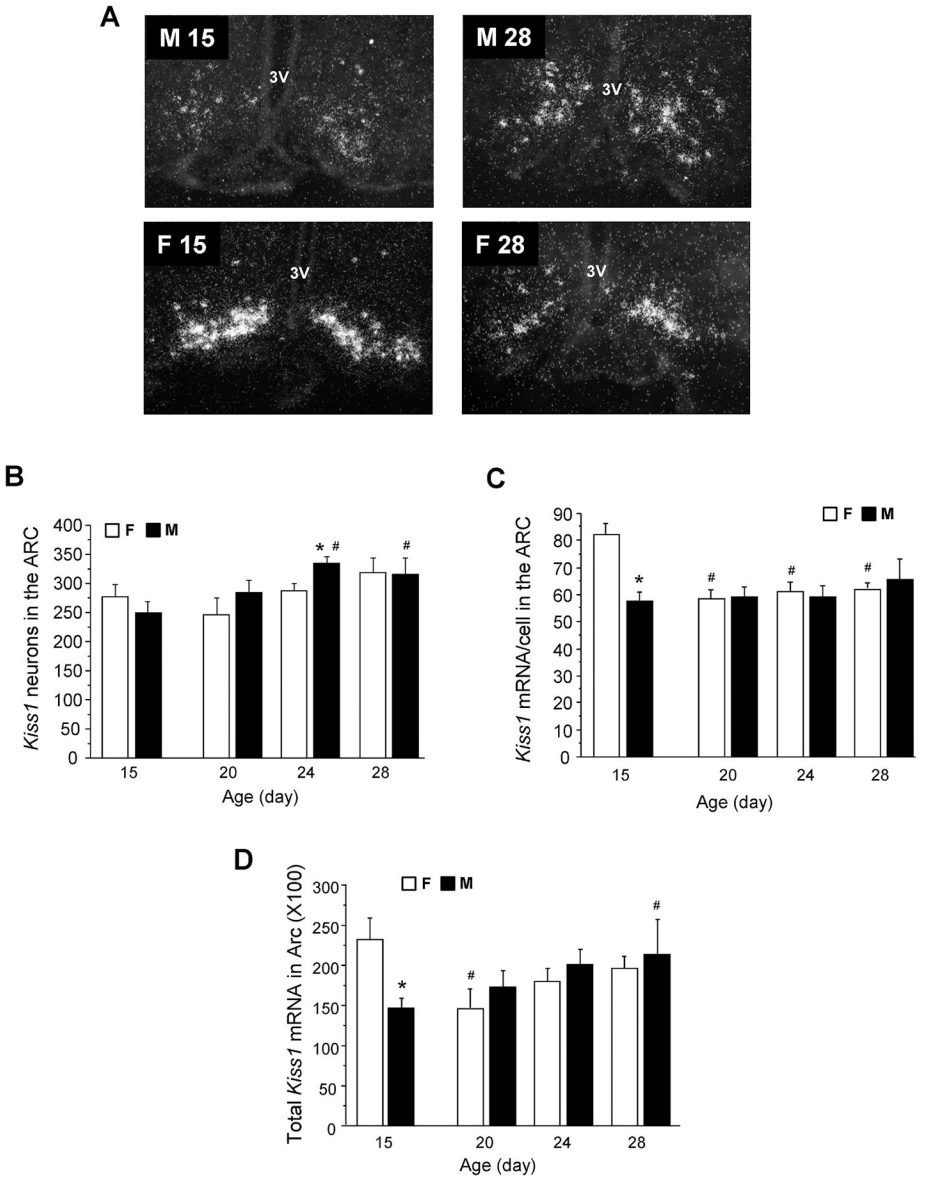

Fig. 2.

Kiss1 expression in the hypothalamic ARC of male and female peri-pubertal mice. **A)** Representative images of *Kiss1* expression, determined by single-label ISH, in the ARC. 3V, third ventricle. **B)** Mean numbers of *Kiss1* neurons in the ARC, **C)** mean *Kiss1* mRNA content per neuron in the ARC, and **D)** mean relative total *Kiss1* mRNA in the ARC region of male and female mice between PND 15 and PND 28. #, $p < 0.05$ vs PND 15 of the same sex. *, $p < 0.05$ vs females of the same age.

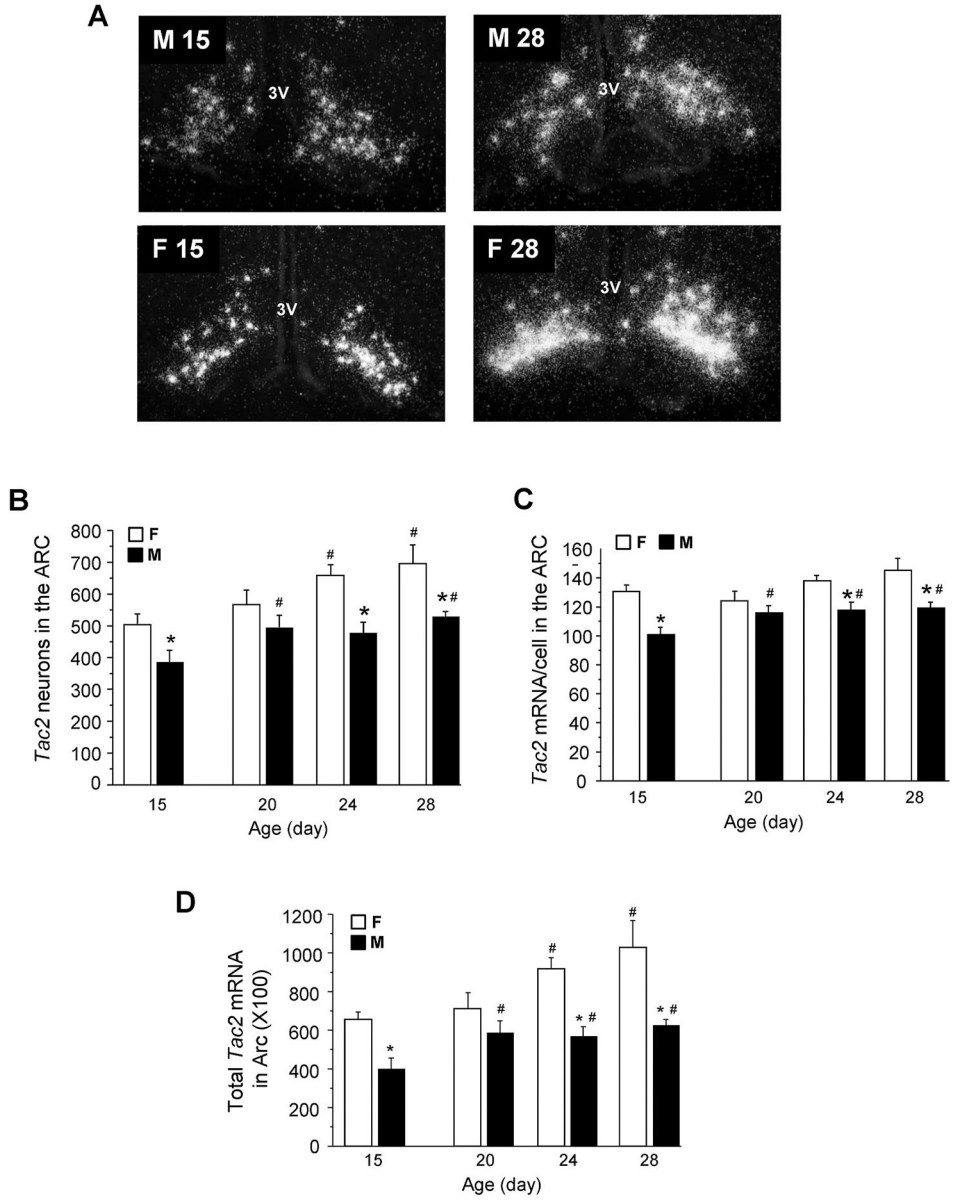

Fig. 3.

Tac2 expression in the hypothalamic ARC of male and female peri-pubertal mice. **A)** Representative images of *Tac2* expression in the ARC. 3V, third ventricle. **B)** Mean numbers of *Tac2* neurons in the ARC, **C)** mean *Tac2* mRNA content per neuron in the ARC, and **D)** mean relative total *Tac2* mRNA in the ARC of male and female mice between PND 15 and PND 28. #, $p < 0.05$ vs PND 15 of the same sex. *, $p < 0.05$ vs females of the same age.

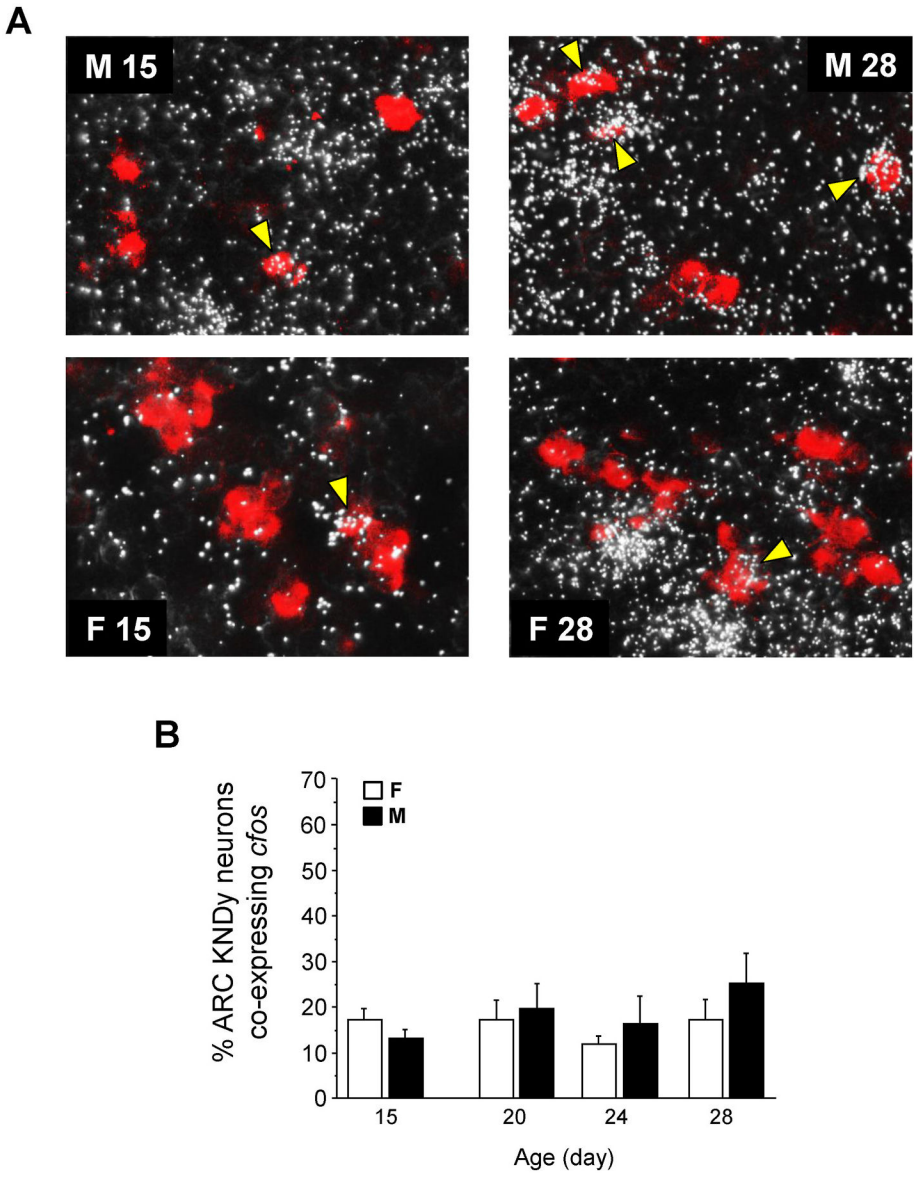

Fig. 4.

KNDy activation during male and female peri-pubertal development. **A)** Representative images of *cfos* mRNA expression (silver grains) in ARC KNDy neurons (red fluorescence) in the ARC of peri-pubertal male and female mice. Yellow arrowheads denote examples of *cfos* co-expressed KNDy neurons. **B)** Mean percent of ARC KNDy neurons expressing *cfos* in males and females between PND 15 and PND 28. #, $p < 0.05$ vs PND 15 of the same sex. *, $p < 0.05$ vs females of the same age.

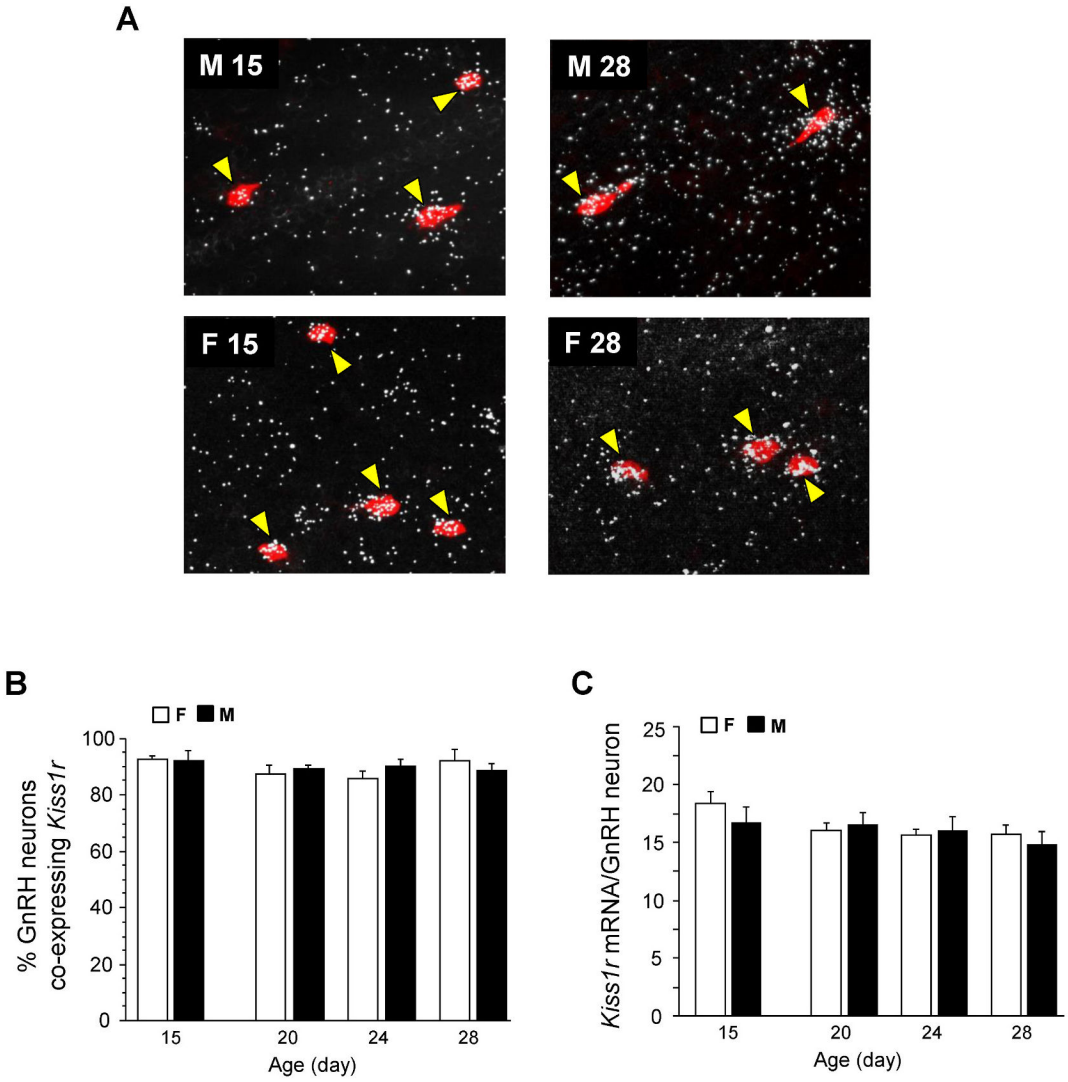

Fig. 5.

Kisspeptin receptor (Kiss1r) levels in GnRH neurons in peri-pubertal males and females. **A)** Representative images of *Kiss1r* mRNA expression (silver grains) in *Gnrh* neurons (red fluorescence) in peri-pubertal mice. **B)** Mean percent of *Gnrh* neurons expressing *Kiss1r* in male and female mice between PND 15 and PND 28. **C)** Mean relative levels of *Kiss1r* mRNA per GnRH neuron in males and females. #, $p < 0.05$ vs PND 15 of the same sex. *, $p < 0.05$ vs females of the same age.

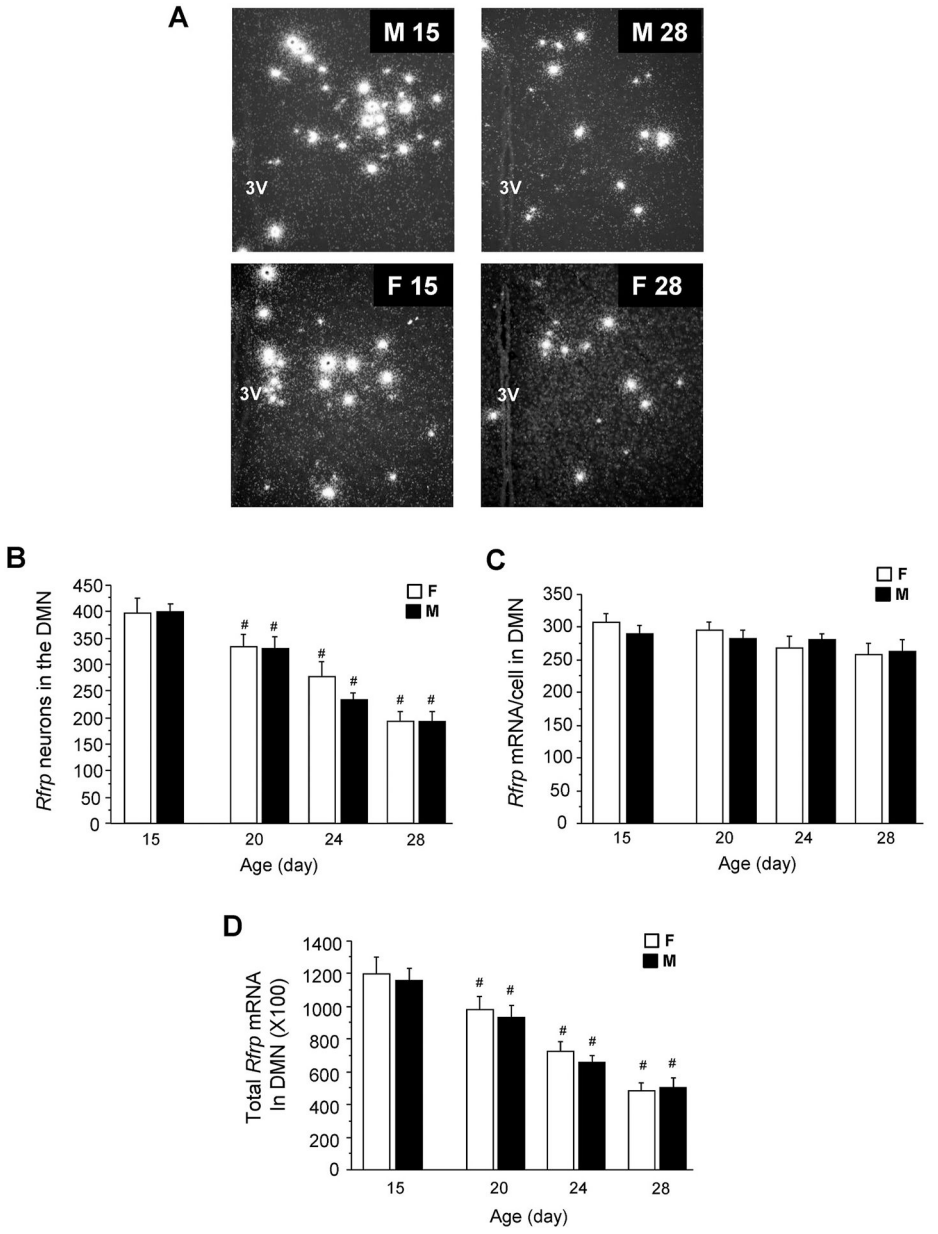

Fig. 6.

Rfrp expression in the DMN of male and female peri-pubertal mice. **A)** Representative images of *Rfrp* expression, determined by radio-labeled ISH, in the DMN. 3V, third ventricle. **B)** Mean numbers of *Rfrp* neurons, **C)** mean *Rfrp* mRNA content per neuron, and **D)** mean relative total *Rfrp* mRNA in of males and females between PND 15 and PND 28. #, $p < 0.05$ vs PND 15 of the same sex. *, $p < 0.05$ vs females of the same age.

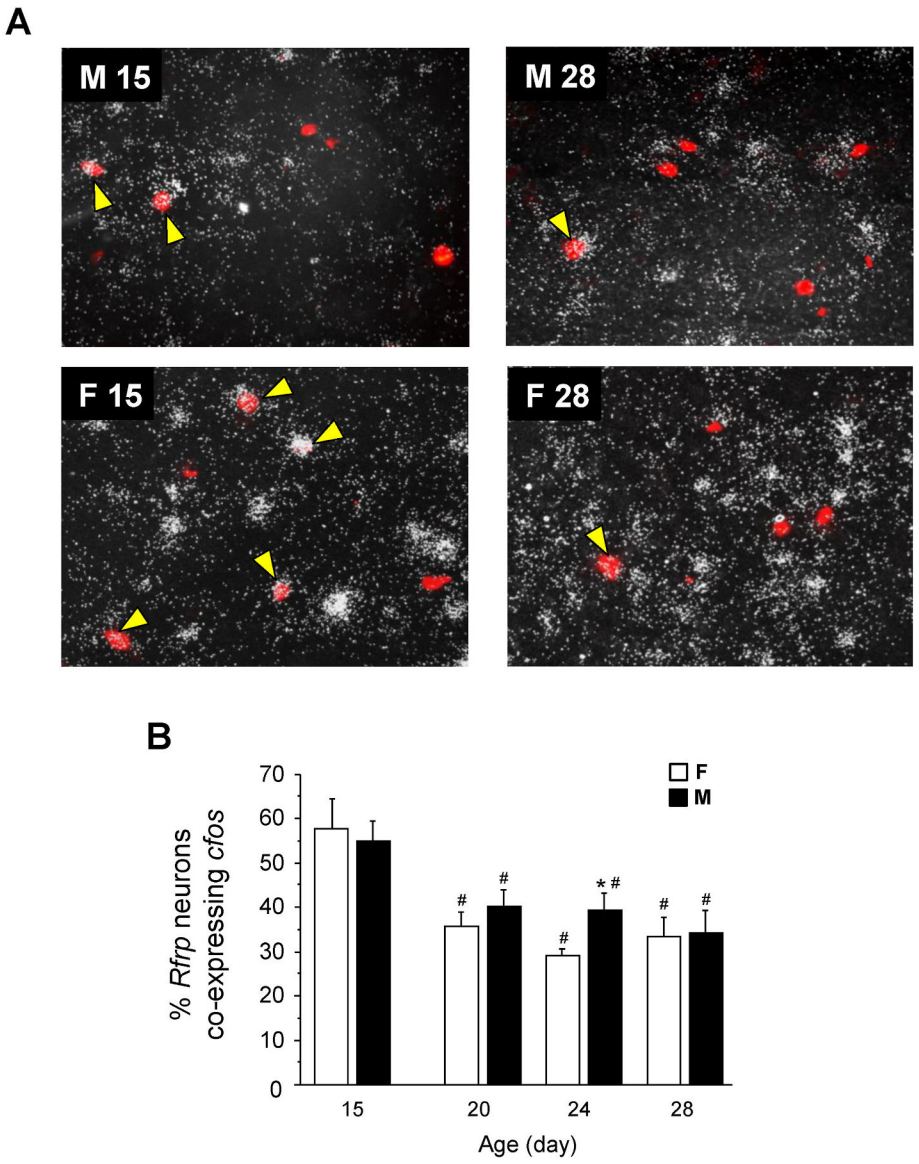

Fig. 7.

Rfrp neuronal activation during male and female peri-pubertal development. **A)** Representative images of *cfos* expression (silver grains) in *Rfrp* neurons (red fluorescence) in the peri-pubertal DMN. Yellow arrowheads denote examples of *Rfrp + cfos* co-expression. **B)** Mean percent of DMN *Rfrp* neurons expressing *cfos* in males and females between PND 15 and PND 28. #, $p < 0.05$ vs PND 15 of the same sex. *, $p < 0.05$ vs females of the same age.

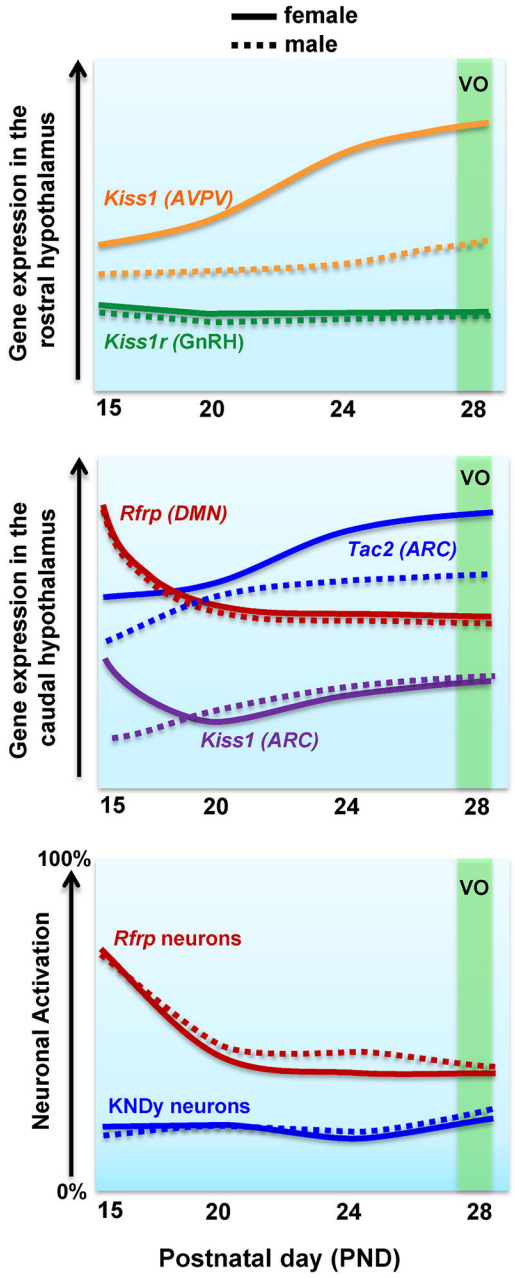

Fig. 8.

Diagram summarizing and comparing the various changes in reproductive gene expression and neuronal activation in different hypothalamic regions during the peri-pubertal transition in male and female mice. In all panels, the male pattern is denoted as dotted lines, females are solid lines. **A, B)** Summary of the developmental changes in neural expression in reproductive genes in the rostral **(A)** or caudal **(B)** hypothalamus during the peri-pubertal transition in males and females. Expression levels on the Y-axis are plotted relative to PND **28** levels. **C)** Summary of developmental changes in neuronal activation (*cfos* colocalization) in hypothalamic reproductive neural populations (ARC KNDy and DMN

RFRP-3 neurons) during the peri-pubertal transition in both sexes. Activation levels are plotted between 0% and 100% co-colocalization for each age.