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Daily successive changes in reproductive gene expression and neuronal activation in the brains of pubertal female mice

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Abstract

Puberty is governed by the secretion of gonadotropin releasing hormone (GnRH), but the roles and identities of upstream neuropeptides that control and time puberty remain poorly understood. Indeed, how various reproductive neural gene systems change before and during puberty, and in relation to one another, is not well-characterized. We detailed the daily pubertal profile (from postnatal day [PND] 15 to PND 30) of neural *Kiss1* (encoding kisspeptin), *Kiss1r* (the kisspeptin receptor), *Tac2* (neurokinin B), and *Rfrp* (RFRP-3, mammalian GnIH) gene expression and day-to-day *c-fos* induction in each of these cell types in developing female mice. *Kiss1* expression in the AVPV/PeN increased steadily over the pubertal transition, reaching adult levels around vaginal opening (PND 27.5), a pubertal marker. However, AVPV/PeN *Kiss1* neurons were not highly activated, as measured by *c-fos* co-expression, at any pubertal age. In the ARC, *Kiss1* and *Tac2* cell numbers showed moderate increases across the pubertal period, and neuronal activation of *Tac2/Kiss1* cells did not vary. Additionally, *Kiss1r* expression specifically in GnRH neurons was already maximal by PND 15 and did not change with puberty. Conversely, both *Rfrp* expression and *Rfrp/c-fos* co-expression in the DMN decreased markedly in the early pre-pubertal stage. This robust decrease of the inhibitory RFRP-3 population may result in diminishing inhibition of GnRH neurons during early puberty. Collectively, our data identify the precise timing of important developmental changes – and in some cases, lack thereof – in gene expression and neuronal activation of key reproductive neuropeptides during puberty, with several changes occurring well before vaginal opening.

Keywords

Puberty; Development; Sexual maturation; Reproduction; Kisspeptin; Kiss1; Kiss1r; RFRP-3; Rfrp; GnIH; Tac2; NKB; GnRH; GPR54

1. Introduction

The onset of puberty is generally defined as the activation of the previously-dormant neuroendocrine reproductive axis (Grumbach, 2002; Ojeda and Skinner, 2006; Plant and Witchel, 2006), reflected by increased secretion of gonadotropin-releasing hormone

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(GnRH). Several upstream hypothalamic circuits have been implicated in the control and modulation of GnRH secretion, but how and when these various reproductive circuits change during development to potentially time and trigger pubertal GnRH secretion is poorly understood (Kauffman, 2010; Ojeda et al., 2006, 2010; Richter, 2006; Tena-Sempere, 2012; Terasawa et al., 2013). Recognized upstream regulators of GnRH neurons range from stimulatory systems, like kisspeptin and neurokinin B (NKB), to inhibitory systems, such as GABA and RFRP-3 (the mammalian homolog of GnIH), not to mention epigenetic factors (Lomniczi et al., 2013; Semaan and Kauffman, 2013). Mutations in several of these systems have resulted in disrupted puberty in humans and animal models. For example, puberty is impaired in humans or mice lacking kisspeptin (encoded by *Kiss1*) or its receptor (Kiss1r) (de Roux et al., 2003; Lapatto et al., 2007; Seminara et al., 2003). Similarly, NKB can stimulate the reproductive axis (Billings et al., 2010; Wakabayashi et al., 2010), and humans with mutations in the gene for NKB, *Tac2*, fail to progress through puberty (Topaloglu et al., 2009; Young et al., 2010).

Developmental alterations in gene expression, protein synthesis, neuronal activation, and secretion of reproductive modulators are likely to be critical aspects driving pubertal progression. However, to date, pubertal changes in most reproductive neural systems have not been examined in sufficient temporal detail. Most studies have only compared gene or protein expression differences *before* and *after* puberty, or in some cases at just one or two single points during the pubertal period (which can last several weeks in rodents and years in primates). Thus, little is known about detailed, sequential changes *during and throughout* puberty. For example, whereas *Kiss1* expression and kisspeptin-immunoreactivity are higher in the AVPV/PeN nucleus of adults compared to prepubertal rodents (Clarkson and Herbison, 2006; Clarkson et al., 2009; Han et al., 2005; Semaan et al., 2010; Takase et al., 2009; Takumi et al., 2011; Walker et al., 2012), the specific developmental pattern during multiple sequential days of the pubertal period itself remains underexplored. Data regarding pubertal changes in the kisspeptin population in the ARC similarly lack detailed temporal resolution, with most studies simply comparing prepubertal versus adult animals without focusing in detail on multiple pubertal ages in between. Moreover, the reported pubertal patterns of *Kiss1* changes (or lack thereof) in the ARC are highly conflicting, often owing to inconsistencies and differences in experimental design, species, sexes, specific age(s) examined, methodology (e.g., qPCR versus in situ hybridization versus immunohistochemistry), and type of measure reported (e.g., cell number versus total expression levels) (Bentsen et al., 2010; Gill et al., 2010, 2012; Han et al., 2005; Lomniczi et al., 2013; Navarro et al., 2012; Takase et al., 2009; Takumi et al., 2011;Walker et al., 2012). The same caveats and limitations, in terms of inconsistencies in ages, sexes, and measures examined, also apply to newer identified reproductive players. For example, *Tac2*, which is coexpressed in virtually all ARC *Kiss1* cells, has been compared between prepubertal and pubertal rodents and found to be higher in the latter (Gill et al., 2012; Navarro et al., 2012), but the temporal resolution of the observed changes were not studied in detail (only every 4–8 days), nor were temporal changes in *Tac2* levels compared to changes in other reproductive genes or pubertal markers (e.g., vaginal opening). Likewise, RFRP-3, an inhibitor of the mammalian reproductive axis (Anderson et al., 2009; Ducret et al., 2009; Kriegsfeld et al., 2006; Wu et al., 2009), has been examined thus far at only sparse stages of

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development, and not yet during puberty. Interestingly, *Rfrp* expression in the mouse brain is higher in juveniles than adults (Poling et al., 2012), but exactly when or in what manner *Rfrp* expression levels change in peri-pubertal animals is unknown.

Previous examinations of reproductive gene differences primarily before and after puberty have left a critical gap regarding information on successive daily changes during and throughout the pubertal period. Furthermore, most previous reports have only studied one protein/gene, and it is therefore unknown how different reproductive factors (kisspeptin, NKB, RFRP-3, etc.) change during puberty in relation to one another. Here, we studied key developmental changes in gene expression and neuronal activation of *multiple* reproductive neural systems (*Kiss1, Tac2, Rfrp*) on a refined temporal level – day-by-day – throughout the pubertal period, and compared these day-by-day changes in one gene system to changes in another. We asked (1) which reproductive genes, or neuronal activation of those neurons, change first during the pubertal process and when? (2) Are specific changes in gene expression or neuronal activation gradual over the pubertal period, or is there an acute, rapid increase (or decrease) on a specific day(s)? (3) What is the temporal relationship between pubertal changes in different gene systems (e.g., between kisspeptin and RFRP-3) or to status of vaginal opening (VO), an oft-used external marker of female "puberty" in rodents?

2. Materials and methods

2.1. Animals

C57Bl6 mice were housed at the University of California, San Diego on a 12–12 light–dark cycle (lights off at 1800 h) with food and water available ad libitum. Female mice, generated from 7 breeder pairs, were weighed at postnatal day (PND) 15 (day of birth = PND 1) and again daily starting at weaning (PND 20). Weaned females were housed in groups of 2–3. Vaginal opening (VO), a commonly-used external marker of puberty, was monitored daily from the time of weaning until sacrifice. Mice were sacrificed at PND 15, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or, for comparison, in adulthood (8–9 weeks; in diestrus stage) (n = 6–8 mice/group). To avoid litter effects, each age group contained mice from at least 3 different litters. All animals were sacrificed between 1100 h and 1300 h, and blood and brains collected. Blood was collected using Microtainer separator tubes and the serum was isolated for hormone measurements. Individual serum blood samples were assayed for LH by the University of Virginia's Ligand Assay Core (Charlottesville, VA), using a sensitive mouse sandwich radioimmunoassay with a limit of detectability of 0.04 ng/ml and intra-assay variation of 7%. Brains were immediately frozen on dry ice and stored at −80 °C. All experiments were conducted with approval of the local Animal Care and Use Committee.

2.2. Single-label in situ hybridization (ISH)

Frozen brains were cut on a cryostat into five sets of 20 μm sections encompassing the entire forebrain and hypothalamus, thaw-mounted onto Superfrost plus slides, and stored at −80 °C. Single-label ISH was performed as previously described (Gottsch et al., 2004; Kauffman et al., 2007; Semaan et al., 2012). Riboprobes utilized were *Kiss1* (Gottsch et al., 2004), *Kiss1r* (Poling et al., 2012), *Tac2* (Kauffman et al., 2009), and *Rfrp* (Poling et al., 2012). Briefly, 1 complete set of slide-mounted sections spanning the entire AVPV/PeN (Plates 26–

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32 in the Franklin and Paxinos mouse stereotaxic atlas), ARC (Plates 41–52), or DMN (Plates 41–51) was fixed in 4% paraformaldehyde, pretreated with acetic anhydride, rinsed in 2× SSC (sodium citrate, sodium chloride), delipidated in chloroform, dehydrated in ethanols, and air-dried. Radiolabeled (P^{33}) antisense riboprobe (0.05 pmol/ml) was combined with yeast tRNA, heat-denatured, added to hybridization buffer, and applied to each slide (100 μl/slide). Slides were put in a humidity chamber at 55 °C for 17 h. The slides were then washed in 4× SSC, placed into RNAse treatment for 30 min at 37 °C, and washed in RNAse buffer without RNase at 37 °C for 30 min. After a wash in 2× SSC at room temperature, slides were washed in 0.1× SSC at 62 °C, dehydrated in ethanols, and air-dried. Slides were then dipped in Kodak NTB emulsion, air-dried, and stored at 4 °C for 6–8 days (depending on the assay) before being developed and cover-slipped.

2.3. Double-label ISH

Double label ISH was performed as described previously (Di Giorgio et al., 2014; Kim et al., 2013; Poling et al., 2012; Robertson et al., 2009). Briefly, slide-mounted brain sections were treated similarly to single-label ISH with the following modifications. Digoxigenin (DIG)-labeled antisense mouse *Gnrh, Kiss1, Tac2*, or *Rfrp* cRNA were synthesized with DIG labeling mix (Roche). Radiolabeled (^{33}P) antisense *c-fos* or *Kiss1r* (0.05 pmol/ml) and DIG-labeled (1:500) riboprobes were combined with tRNA, heat denatured, and dissolved together in hybridization buffer. The probe mix was applied to slides (100 μl/slide) and hybridized at 55 °C overnight. After the 62 °C washes on day 2, slides were incubated in blocking buffer for 1 h at room temperature and then incubated overnight at room temperature with anti-DIG antibody conjugated to alkaline phosphatase [(Roche) diluted 1:500]. Slides were then washed with Buffer 1 and incubated with Vector Red alkaline phosphatase substrate (Vector Labs, CA) for 1 h at room temperature. The slides were then air-dried, dipped in emulsion, stored at 4 °C, and developed 7–10 days later, depending on the assay.

2.4. Quantification and statistical analysis

ISH slides were analyzed with an automated image processing system (Dr. Don Clifton, University of Washington) by a person blind to the treatment group. For single-label experiments, the software counted the number of silver grain clusters representing cells, as well as the number of silver grains in each specific cell cluster (a semi-quantitative index of mRNA content per cell) (Chowen et al., 1990). A relative measure of total mRNA for a specific gene in a brain area was determined by multiplying the total number of cells in that region by the relative amount of mRNA content per cell (Kim et al., 2013; Navarro et al., 2011). Cells were considered positive when the number of silver grains in a cluster exceeded that of background by threefold. For double-label assays, red fluorescent DIG-containing cells were identified under microscopy and the grain-counting software quantified the number of silver grains overlying each cell. Signal-to-background ratios for individual cells were calculated, and a cell was considered double-labeled if its ratio was >3 (Di Giorgio et al., 2014; Kauffman et al., 2014; Navarro et al., 2011).

For each gene, we quantified total cell number, relative mRNA level per cell, and total relative levels of mRNA in the entire brain region, as any one or more of these different

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measures might change during puberty. All data are expressed as the mean ± SEM for each group. Differences in group means were assessed via overall ANOVA or 2-way ANOVA with post-hoc analysis determined by Fisher's LSD test. Statistical significance was set at $p < 0.05$. Correlation analysis between genes was performed via determination of Pearson's correlation coefficient and analyzed for significance with Fisher's r to z test ($p < 0.05$).

3. Results

3.1. Somatic and hormonal measures before and during puberty

Body weight and the occurrence of VO (an external morphological indicator of female puberty) were measured before and during the pubertal transition (at PND 15 and every day from PND 20 to PND 30). Body weight steadily increased from PND 15 through PND 30, and the average age of VO was ~PND 28 (range of VO occurrence: PND 26–30; Fig. 1). LH levels in serum increased from PND 15 to PND 20 and remained elevated thereafter throughout the pubertal period ($p < 0.05$; Fig. 1).

3.2. AVPV/Pen Kiss1 expression in female mice before and during puberty

Previous developmental studies measured *Kiss1*/kisspeptin expression across largely spaced intervals of time, either before and after puberty or on just 1 or 2 days of the entire pubertal period. Here we examined a much more detailed, day-by-day pubertal profile of *Kiss1* mRNA expression, looking separately at the AVPV/PeN and ARC populations. In the AVPV/PeN, *Kiss1* neuron cell number increased steadily and consistently throughout the peripubertal ages examined, peaking to adult levels around the time of mean VO (~PND 27; Fig. 2). The relative level of *Kiss1* mRNA/cell and total *Kiss1* mRNA levels in AVPV/PeN also increased steadily across the pubertal period with a similar pattern (Fig. 2). Restricting analysis to just the age range when VO was observed, we determined that, independent of age, the levels of AVPV/PeN *Kiss1* expression did not differ between females that had or had not already displayed VO at the time of sacrifice (termed pre-VO and post-VO, respectively; Fig. 2).

3.3. Developmental profile of Kiss1 and Tac2 expression in the ARC before and during puberty

Prior data on rodent ARC *Kiss1* expression during peri-pubertal stages are fairly inconsistent, confounded by differing or sporadic ages of analyses, with some reports of pubertal increases in *Kiss1* expression and other reports of no changes (Gill et al., 2010; Lomniczi et al., 2013; Navarro et al., 2012; Takase et al., 2009; Takumi et al., 2011). Here, we found that the number of *Kiss1* neurons in the ARC rose moderately and significantly over the pubertal transition, showing initial increases around PND 24 and reaching adult levels around the time of VO, with older pubertal ages (PND28–30) being significantly higher than earlier pubertal ages (PND 20–22) ($p < 0.05$; Fig. 3A and B). Intriguingly, unlike the pattern of cell number, the relative amount of *Kiss1* mRNA per cell in the ARC was highest at PND 15, and dropped significantly by PND 20 ($p < 0.01$), remaining unchanged at all pubertal ages afterward (Fig. 3C). Levels of total relative *Kiss1* mRNA in the ARC region were also high on PND 15 and dropped significantly by PND 20 ($p < 0.05$; Fig. 3D); total ARC *Kiss1* levels then remained at this lower level from PND 20 to PND 25,

after which they increased again and were significantly higher during most of the VO period ($p < 0.05$; Fig. 3D). When looking at females sacrificed just during the VO period (~PND26–29) and comparing pre-VO versus post-VO status, ARC *Kiss1* cell number showed a strong trend for being higher after VO than before VO that just missed statistical significance ($p = 0.054$), Fig. 3E). *Kiss1* mRNA/cell and total ARC *Kiss1* levels did not differ between pre-VO and post-VO during the ages of the VO period (Fig. 3F and G).

We next analyzed pubertal changes in *Tac2* mRNA, which encodes the reproductive neuropeptide NKB and is coexpressed in virtually all ARC *Kiss1* cells (often termed KNDy cells). As with ARC *Kiss1* cell number, we found a modest gradual increase in *Tac2* cell number in the ARC throughout the peri-pubertal ages examined ($p < 0.05$; Fig. 4B), with cell number reaching adulthood levels around PND 24. Similar gradual increases in the level of *Tac2* mRNA per cell were also observed, reaching adult levels around PND 28 ($p < 0.05$; Fig. 4C). Likewise, total *Tac2* mRNA levels in the ARC nucleus demonstrated a moderate increase at later pubertal ages compared to younger juvenile and peripubertal animals (PND 15, 20, 21, 22) ($p < 0.05$; Fig. 4D), culminating in adult levels around PND 24. During just the ages of the VO period, *Tac2* cell number and total *Tac2* expression were significantly higher in post-VO females versus pre-VO females, independent of age during this particular period ($p < 0.05$; Fig. 4E and G).

3.4. Kiss1 neuronal activation in female mice before and during puberty

The pubertal profile of *Kiss1* neuronal activation has not yet been determined for any species, and could change independent of pubertal changes in gene expression. Whereas *Kiss1* levels in the AVPV/PeN increased markedly throughout the pubertal period (Fig. 2), *cfos-Kiss1* coexpression in the AVPV/PeN was very minimal at all peripubertal ages, being typically 5% on most days examined (Fig. 5B). Moreover, no differences in *cfos/Kiss1* co-expression in the AVPV/PeN were noted in females of differing VO status during the VO period (Fig. 5D).

Similar analyses were performed in the ARC for *Kiss1* ("KNDy") neuronal activation by measuring the numbers of these ARC neurons co-expressing *cfos* at each age. A moderate number of ARC *Kiss1* cells co-expressed *cfos* at any given age, greater than that observed in the AVPV/PeN *Kiss1* population. However, the degree of *Kiss1* neuronal activation in the ARC did not fluctuate significantly throughout the peri-pubertal ages examined, remaining around 18–20%, nor was there any alteration in the level of ARC *Kiss1-cfos* co-expression based on VO status (Fig. 5C and E).

3.5. Kisspeptin receptor expression exclusively in GnRH neurons in mice before and during puberty

An important aspect of kisspeptin's potential effects on puberty is its ability to activate GnRH neurons via signaling through Kiss1r. We therefore examined the successive day-by-day pattern of *Kiss1r* mRNA levels exclusively in GnRH neurons across the peripubertal transition. On PND 15, the percent of *Gnrh* neurons co-expressing *Kiss1R* was maximal and already at adult levels (Fig. 6); *Kiss1r-Gnrh* coexpression levels did not change at all across the pubertal period or in relation to VO (Fig. 6). In addition, the relative levels of *Kiss1r*

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mRNA specifically in GnRH neurons were not different at any age between PND 15 and PND 30 and were identical during all pubertal ages to adulthood levels (Fig. 6).

3.6. Peripubertal decreases in Rfrp expression and neuronal activation in the DMN of female mice

The neuropeptide RFRP-3 can inhibit the reproductive axis (Anderson et al., 2009; Ducret et al., 2009), and *Rfrp* cell number is lower in adulthood than in juvenile mice (Poling et al., 2012). We therefore determined exactly when this developmental reduction occurs and whether *Rfrp* neuronal activity was similarly changed during puberty. Interestingly, both the number of *Rfrp* neurons and total *Rfrp* mRNA in the DMN region were highly elevated on PND 15 and dropped significantly around PND 20–21 to adult levels ($p < 0.05$; Fig. 7B and D). The relative level of *Rfrp* mRNA in each cell remained generally constant (Fig. 7C). During the VO period (~PND26–29), females who had already undergone VO demonstrated significantly lower *Rfrp* cell number and total *Rfrp* mRNA levels versus pre-VO females, independent of age ($p < 0.01$; Fig. 7E and G).

In addition to the marked peripubertal decreases in *Rfrp* expression levels, the activational status of *Rfrp* neurons, as measured by *c-fos* co-expression, also decreased substantially between PND 15 and PND 20 ($p < 0.05$; Fig. 8A and B), remaining relatively constant thereafter with a slight non-significant trend for further decreases in the later pubertal period.

3.7. Correlation analysis of peri-pubertal reproductive gene expression

To further analyze the relationship between the various brain measures studied across the pubertal transition in our female mice, we performed a correlation analysis of neural reproductive gene expression and neuronal activation during the pubertal period. Table 1 depicts the Pearson correlation coefficients, demonstrating several significant correlations between the various gene systems during the pubertal period. Some pubertal gene measures were strongly negatively correlated (e.g., *Rfrp* and AVPV/PeN *Kiss1* cells; *Rfrp* neuronal activation levels and *Tac2* cell number, etc), whereas others were strongly positively correlated (e.g., ARC *Kiss1* cells and ARC *Tac2* mRNA; *Rfrp* mRNA and *Rfrp* neuronal activation, etc.) (Table 1).

4. Discussion

Several hypothalamic circuits have been implicated in governing the timing and progression of maturation of the reproductive axis, converging on GnRH neurons to trigger puberty onset. The kisspeptin system, along with NKB, is strongly implicated in pubertal development in mammals, including humans (de Roux et al., 2003; Lapatto et al., 2007; Seminara et al., 2003; Topaloglu et al., 2009, 2012; Young et al., 2010). Yet, despite their proposed involvement in the developmental maturation of reproductive capabilities, how these various reproductive neural systems change developmentally has primarily been compared before and after puberty (Clarkson et al., 2009; Gill et al., 2012; Navarro et al., 2004; Poling et al., 2012; Takumi et al., 2011), with far less analysis on their changes during and throughout puberty, either on their own or in relation to one another. Here, we provide

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the first detailed, day-by-day peripubertal gene expression profiles in developing female mice, as well as neuronal activation patterns via *c-fos* co-expression, in several key neural populations influencing GnRH.

4.1. Kisspeptin in the AVPV/PeN

Sexual maturation is impaired in humans and mice with mutations in the *Kiss1r* or *Kiss1* genes (de Roux et al., 2003; Seminara et al., 2003; Topaloglu et al., 2012), and exogenous kisspeptin administered to prepubertal animals induces various aspects of puberty (such as increased LH secretion or VO) (Navarro et al., 2004; Shahab et al., 2005). Although in vivo secretion of neuropeptides in mice, especially at younger ages, is nearly impossible to study, work performed in non-human primates demonstrated increased kisspeptin secretion at later stages of puberty versus before puberty (Guerriero et al., 2012). Moreover, ex vivo kisspeptin secretion from hypothalamic explants is higher in female pubertal monkeys compared to juveniles (Keen et al., 2008). However, the neuroanatomical source of this pubertal kisspeptin secretion (AVPV/PeN versus ARC) remains unclear, as does the timing of endogenous kisspeptin's developmental onset. Previous studies in mice indicated that AVPV/PeN *Kiss1* mRNA and kisspeptin protein are undetectable prior to PND 10 and 15, respectively (Clarkson et al., 2009; Semaan et al., 2010), and then increase from PND 15 to adulthood, as assessed every 5 days of age (Clarkson et al., 2009). As in mice, AVPV/PeN kisspeptin cells in rats are not detectable on or before PND 8, but are present by the next age examined (PND 22), and are even higher in adulthood (Takumi et al., 2011). Yet in these previous studies, it remained unclear if AVPV/PeN kisspeptin/*Kiss1* gradually increases across puberty or if this increase happens quickly at a potentially important age or pubertal stage. Likewise, whether the increase in AVPV/PeN kisspeptin/*Kiss1* occurs before, at, or after changes in other genes was not previously determined. Using a detailed, day-by-day pubertal analysis of *Kiss1* mRNA expression, our present findings demonstrate that *Kiss1* cell number, *Kiss1* mRNA per cell, and total *Kiss1* mRNA levels in the AVPV/PeN markedly, but consistently and gradually, increase from PND 15 through PND 30. The daily increases in AVPV/PeN *Kiss1* expression occur well before VO and finally resembled adulthood levels around the period of VO (by PND 26–29, depending on the specific measure). The AVPV/PeN kisspeptin system has been implicated in the preovulatory LH surge in adult females, but at present has not yet been linked functionally to puberty. Thus, whether these robust pubertal increases in AVPV/PeN *Kiss1* levels are involved in puberty onset or progression currently remains unknown. Interestingly, while AVPV/PeN *Kiss1* levels increased consistently with age, AVPV/PeN *Kiss1* did not vary with VO status at ages specifically during the VO period, suggesting that *Kiss1* increases at that particular period are age-dependent and may not relate to puberty or VO status.

To provide additional insight, we also examined whether the neuronal activation of the AVPV/PeN *Kiss1* population also changed with pubertal status. At all developmental ages examined, *c-fos* co-expression in *Kiss1* AVPV/PeN cells was very minimal and did not change over the pubertal period. Thus, despite dramatic increases in *Kiss1* levels throughout the pubertal transition, AVPV/PeN *Kiss1* neurons do not appear to be activated, as reflected by cfos induction, during this developmental period, at least at the specific times of day examined. This lack of AVPV/PeN *Kiss1* neuronal activation at early and mid-pubertal ages

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is in alignment with the proposed role of these particular *Kiss1* neurons not as drivers of pulsatile GnRH but as generators of the preovulatory GnRH/LH surge, an event which does not first occur until the very end of puberty, typically signifying attainment of reproductive capability. Of note, we only studied *c-fos* and therefore cannot rule out that other less commonly-used markers of neuronal activation might reveal a different result with respect to AVPV/PeN *Kiss1* neuronal activation.

4.2. Kisspeptin and NKB in the ARC

Unlike in the AVPV/PeN, ARC *Kiss1* expression is readily detectable prenatally and at birth in rodents, and continues to be expressed throughout postnatal development (Cao and Patisaul, 2011; Kumar et al., 2014; Poling and Kauffman, 2012). However, previous data regarding peripubertal changes in ARC *Kiss1* gene expression are either lacking, incomplete, or conflicting. Some studies report small increases in ARC *Kiss1* expression around early puberty (Bentsen et al., 2010; Takase et al., 2009) and a more dramatic pubertal increase has also recently been reported (Lomniczi et al., 2013). Conversely, other studies have reported no major differences in ARC *Kiss1* levels between juvenile and adult rodents (Gill et al., 2010; Han et al., 2005; Navarro et al., 2012), leaving the issue unresolved. Here, our detailed day-by-day assessment determined that between PND 20 and PND 30, the number of ARC *Kiss1* neurons and total levels of *Kiss1* mRNA in the ARC increased moderately, with significant increases first evident around PND 24–26 and the overall increase from PND 20 to PND 30 being ~25%. However, interestingly, the amount of *Kiss1* mRNA per cell in the ARC was highest at PND 15; between PND 15 and PND 20, the level of *Kiss1* mRNA per cell dropped ~27% and, correspondingly, the total *Kiss1* mRNA in the ARC also decreased during this pre-pubertal stage. This decrease in *Kiss1* levels per cell may be due in part to increasing estradiol levels during puberty, since estradiol is known to repress *Kiss1* expression in the ARC (i.e., negative feedback) (Smith et al., 2005). It is not clear if or how the pre-pubertal decrease in ARC *Kiss1* levels relates to the triggering pubertal onset, but suggests that enhanced kisspeptin synthesis per cell occurs well before the pubertal period. Intriguingly, this matches the fact that female mice lacking estradiol negative feedback in kisspeptin cells initiate very early VO, evident around PND 15 (Mayer et al., 2010).

Kiss1 neurons in the ARC highly co-express *Tac2*, encoding NKB (Goodman et al., 2007; Navarro et al., 2009; Rance and Bruce, 1994) (the so-called KNDy neurons). NKB can stimulate LH secretion, likely by triggering kisspeptin secretion (Billings et al., 2010; Navarro et al., 2009;Wakabayashi et al., 2010), and mutations in the NKB system impair puberty (Topaloglu et al., 2009; Young et al., 2010). Recently, it was shown that *Tac2* mRNA in the MBH of mice (measured with qPCR of MBH dissections) is higher in pubertal than prepubertal mice (Gill et al., 2012), and another study in female rats reported increased MBH *Tac2* levels before puberty and in the later pubertal period, but no changes in between (measured every 4–6 days) (Navarro et al., 2012). In both cases, the temporal resolutions of these potentially important developmental increases were not studied in detail, nor were *Tac2* levels compared to changes in other genes or pubertal markers. Besides the lack of temporal resolution, a further limitation of these previous findings was that analyses were done on MBH brain dissections, which encompass portions of neighboring non-ARC brain

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regions which also express *Tac2*, thereby lacking anatomical resolution. In our present study, we used ISH to focus solely on the ARC *Tac2* population (i.e., KNDy neurons) and documented a steady, moderate increase in *Tac2* cell number and total *Tac2* mRNA levels in the ARC across the peripubertal period, being ~30–35% higher at later pubertal ages (PND 28–30) than at PND 15. Between PND 20 and PND 30, total ARC *Tac2* mRNA expression increased by ~30%, which is similar in magnitude to a previous report studying just those two ages using qPCR (Gill et al., 2012). In the present study, an additional comparison of mice before and after VO status, during just the age period when VO is observed, demonstrated notable *age-independent* increases in both ARC *Tac2* and *Kiss1* cell numbers, as well as total *Tac2* expression, which correlated with VO status. This suggests that elevations in *Tac2* and *Kiss1* in the ARC may be a good indicator of VO status and, hence, pubertal progress. Unlike *Tac2* and *Kiss1* gene expression, neuronal activation of ARC KNDy cells did not change significantly over the pubertal period. However, overall neuronal activation levels were notably higher in the ARC *Kiss1* versus AVPV *Kiss1* cells at all pubertal ages analyzed, suggesting that ARC *Kiss1* cells exhibit more neural activity in general at this peripubertal period than AVPV *Kiss1* cells, regardless of gene expression levels. The functional significance of this regional *Kiss1* difference in neuronal activation is not yet known, but may relate to the proposed involvement of the ARC, but not AVPV/PeN, in driving GnRH pulses (which increase at puberty).

4.3. Kisspeptin receptor in GnRH neurons

In addition to *Kiss1*, changes in the kisspeptin receptor, Kiss1r, may also be involved in pubertal maturation, though this has received little attention. Although kisspeptin administered to prepubertal rodents and monkeys induces various aspects of precocious puberty (Navarro et al., 2004; Shahab et al., 2005), low kisspeptin doses are less effective at stimulating gonadotropin secretion and GnRH neuronal firing activity in juvenile than adult rodents (Castellano et al., 2006; Han et al., 2005), suggesting that kisspeptin has a reduced ability to activate the GnRH system before puberty. Moreover, in rats of both sexes and female monkeys, though not male mice, hypothalamic *Kiss1r* expression is higher in adulthood than in juveniles (Han et al., 2005; Navarro et al., 2004; Shahab et al., 2005). However, in almost all cases, *Kiss1r* was measured in whole hypothalamus, rather than in specific cell-types, preventing identification of which specific neuronal populations the changes occur in. Here, we determined that the level of *Kiss1r* expression specifically in *Gnrh* neurons in female mice is already at maximal adult levels by PND 15 and does not vary during any stage of the pubertal transition. A recent mouse study utilizing lacZ expression as a proxy for Kiss1r reported that at some undetermined point between PND 5 and PND 20, there was a significant increase in the number of GnRH cells expressing Kiss1r (Herbison et al., 2010). Our data suggest that this previously-reported developmental increase in Kiss1r-GnRH coexpression occurs *before* PND 15, because no change in the percent of *Kiss1r-Gnrh* colocalization was observed in our mice after this age.

We also found that, like the prevalence of *Kiss1-Gnrh* colocalization, the relative amount of *Kiss1r* mRNA in *Gnrh* neurons showed minimal variation during the pubertal period and was virtually equivalent to adult levels at all pubertal ages examined. This suggests that the ability for kisspeptin to signal to its receptor is already present and maximal well before

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external markers of puberty (such as VO) occur. Previous studies that reported increases in hypothalamic *Kiss1r* levels between juvenile life and adulthood measured *Kiss1r* in whole hypothalamus and therefore combined multiple hypothalamic regions and cell-types that may express this receptor. Our present ISH data allow for cell-type specificity and clearly demonstrate no significant pubertal increases in *Kiss1r* specifically in GnRH neurons. This indicates that the previously-reported developmental increase occurs in non-GnRH cells, the identity and function (reproductive or otherwise) of which remain to be determined.

4.4. RFRP-3 in the DMN

In contrast to kisspeptin and NKB, which stimulate the reproductive axis, RFRP-3 is characterized as an inhibitor of reproductive hormone secretion (Anderson et al., 2009; Ducret et al., 2009). Because puberty onset could include modulation of an upstream "brake" on GnRH secretion, we assessed whether *Rfrp* neurons (which are located exclusively in the DMN) show notable changes before or during puberty. Neural *Rfrp* expression in mice was previously examined at a few developmental ages, with total *Rfrp* cell number being higher in juveniles than adults (Poling et al., 2012), but it was unknown exactly when and how *Rfrp* levels change in post-juvenile (i.e., pubertal) mice. Strikingly, we found that both the number of *Rfrp* neurons and total *Rfrp* mRNA dropped significantly, by 30% and 40%, respectively, between PND 15 and PND 21, after which they stabilized at adult levels. This large decrease in *Rfrp* expression over the course of a few days may point to this particular peripubertal stage as a critical period for a reduction of RFRP-3-mediated inhibition of GnRH or kisspeptin neurons (Poling et al., 2013). Notably, this drop in *Rfrp* expression occurs almost a week prior to VO, suggesting that hypothalamic changes in reproductive circuits occur well before external physical signs of pubertal onset. However, interestingly, during the VO period itself, mice that had already undergone VO also had significantly lower *Rfrp* expression, both in cell number and total mRNA levels (~20% reduction for each), than mice of the same ages that had not yet shown VO. Moreover, unlike the AVPV/PeN and ARC *Kiss1* cells, the *Rfrp* population exhibited a large peripubertal change in neuronal activation. In juvenile animals, the number of *Rfrp* cells co-expressing *c-fos* was dramatically higher – by nearly twofold – than on any subsequent pubertal age examined. What is causing the *Rfrp* neuron activation at PND 15 but not at older pubertal ages is currently unknown, but may potentially comprise an integral part of the pubertal process. However, at present, very little – if anything – is known regarding the identity of upstream or internal factors that regulate *Rfrp* neurons.

The observed peri-pubertal decreases in *Rfrp* levels and *Rfrp* neuronal activation accord with the proposed role of RFRP-3 as an inhibitor of the reproductive axis (Anderson et al., 2009; Ducret et al., 2009), and may indicate that RFRP-3 signaling is reduced prior to, or at, the onset of puberty in order to disinhibit the reproductive axis. Indeed, it has been proposed that upstream networks may control puberty through the relief of inhibition on stimulatory factors, like kisspeptin and NKB, to allow GnRH secretion to be enhanced at puberty (Kauffman, 2010; Lomniczi et al., 2013; Tena-Sempere, 2012; Terasawa et al., 2013). Based on our current findings, RFRP-3 signaling may be one possible aspect of inhibitory control during puberty onset. Despite this possibility, a recent report suggested that RFRP-3 signaling via GPR147 is not crucial for puberty onset, as GPR147 KO mice exhibited

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normal puberty onset (Leon et al., 2014). However, RFRP-3 can also bind the receptor GPR74, which was not only intact in those KO mice but actually upregulated in some tissues, perhaps providing compensatory pathways and maintaining functional RFRP-3 signaling. Thus, whether RFRP-3 signaling plays a role in pubertal timing still remains to be determined.

4.5. General considerations and conclusions

The present study identifies several notable changes in gene expression and neuronal activation at various stages of the pubertal period (summarized in Fig. 9). The timing of the observed changes is interesting for several reasons. First, not all the changes occurred at the same time or over the same number of days, indicating differential regulation of these various reproductive systems during the peripubertal period. For example, RFRP-3 changed dramatically early in the peripubertal period but not later on, whereas ARC *Kiss1* and *Tac2* changed very gradually and constantly throughout the pubertal period. *Kiss1* in the AVPV/PeN, like the ARC, changed throughout the entire period but on a much larger scale day to day. Second, many of the observed changes, including *Rfrp* and AVPV/PeN *Kiss1*, occurred well before VO (Fig. 9), an external morphological marker of puberty. Indeed, the *Rfrp* changes occurred over a week before mean VO, and likewise, AVPV/PeN *Kiss1* was already showing notable increases a week before VO. These observations support the idea that pubertal changes in the brain may actually occur much earlier than VO, which is merely a morphological marker of "sufficient" estradiol exposure and, as such, is not a good indicator of puberty *onset*, but rather of pubertal occurrence or progression. Though many studies of puberty in rodent models use VO as an indicator of the "onset of puberty", it appears that neuroendocrine puberty in the brain has likely begun well before the VO event.

Whether any of the notable changes observed in the present study actually reflect an involvement in the pubertal mechanism versus other important physiological processes remains to be determined. Likewise, it is unknown if some of the alterations observed are secondary responses to pubertal changes in gonadal sex steroids, which presumably rise during the pubertal period, as demonstrated in other rodents. In particular, the large AVPV/PeN *Kiss1* increases are likely due, at least in part, to rising estradiol levels during pubertal development, as AVPV/PeN *Kiss1* expression can be strongly upregulated by activational effects of sex steroids (Smith et al., 2005). However, current mouse estradiol assays are not sensitive enough to detect very low levels of estradiol, especially at young ages (Kauffman, unpublished observations), and we were therefore unable to correlate serum estradiol in our pubertal mice to changing gene expression. Regardless, many of the observed pubertal changes in gene expression cannot be solely due to sex steroids, as the different genes changed with different patterns, magnitudes, and time-courses (or in some cases, did not change at all), despite being exposed to the same hormonal milieu within each animal. In fact, interestingly, the ARC *Kiss1* and *Tac2* systems increased over the pubertal period, despite presumably rising sex steroid levels (which normally inhibit these two genes, at least in adults). In addition, we note that all animals in the present study were sacrificed during a 2-hour time window during the day (from 11 am to 1 pm), and gene expression levels and neuronal activation of these various reproductive populations may change differently (or not at all) at other circadian times outside of this period. Given the large scale

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of the present study, it was not logistically possible to include analyses at multiple time points each day.

Future analyses of the same neural genes in pubertal males will be critical. Indeed, it will be informative to determine what commonalities and differences exist between male and female reproductive genes, especially given known sex differences in both normal puberty onset (earlier in females than males) and pubertal disorders in humans (precocious puberty is more common in females, delayed puberty more prevalent in males). Moreover, our study focused on gene expression and neuronal activation, but did not measure neuropeptide protein levels. Future studies can perform similar large-scale analysis of protein levels across puberty to complement and extend our mRNA findings.

In summary, we report that multiple reproductive genes are in flux during the peripubertal and pubertal period, with marked and continual increases in AVPV/PeN *Kiss1* expression, smaller gradual in ARC *Kiss1* and *Tac2* expression, and a more rapid and sizable drop in *Rfrp* expression and neuronal activation in the early portion of the peripubertal period. The observed increases in *Kiss1* and *Tac2* may relate to increased stimulation of the maturing reproductive axis, whereas the reduction in *Rfrp* levels and neuronal activation may reflect disinhibition of reproductive circuits to facilitate puberty onset. Several of these changes occurred well before external morphological signs of puberty, though interestingly, many of the genes changed on quite different timescales and patterns than each other.

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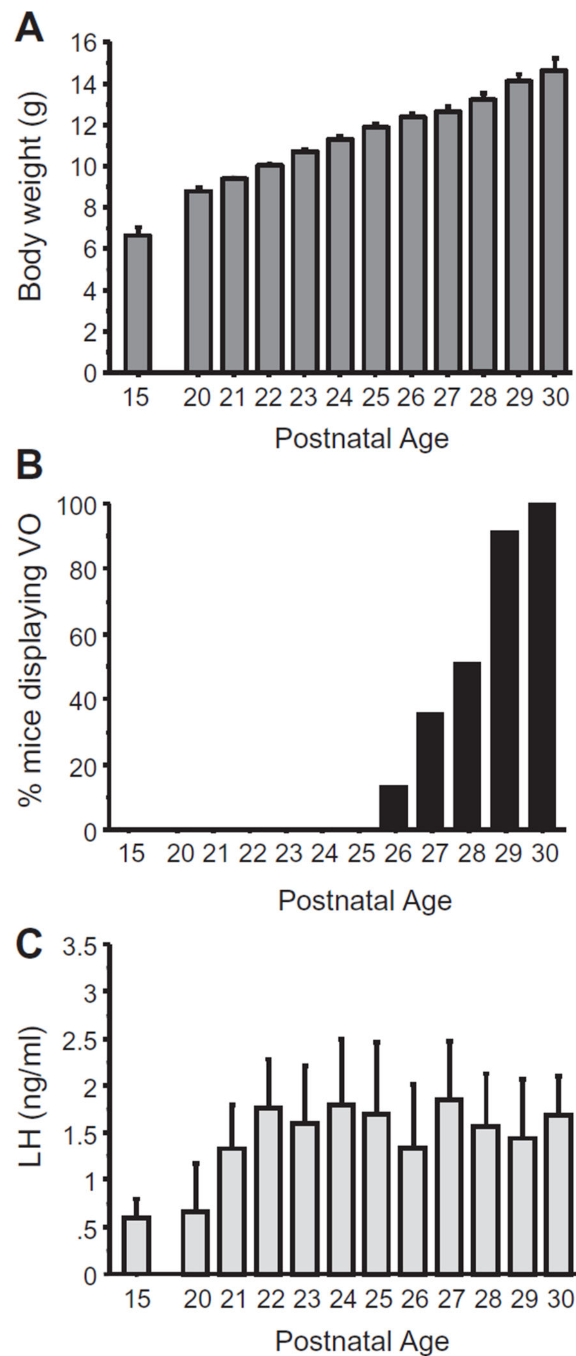

Fig. 1.

Somatic and endocrine measures in peri-pubertal female mice. (A) Body weight of female mice over peri-pubertal development. (B) % of female mice displaying vaginal opening (VO), an external marker of puberty in rodents, at various developmental ages. (C) Mean serum LH levels in peri-pubertal females at different ages.

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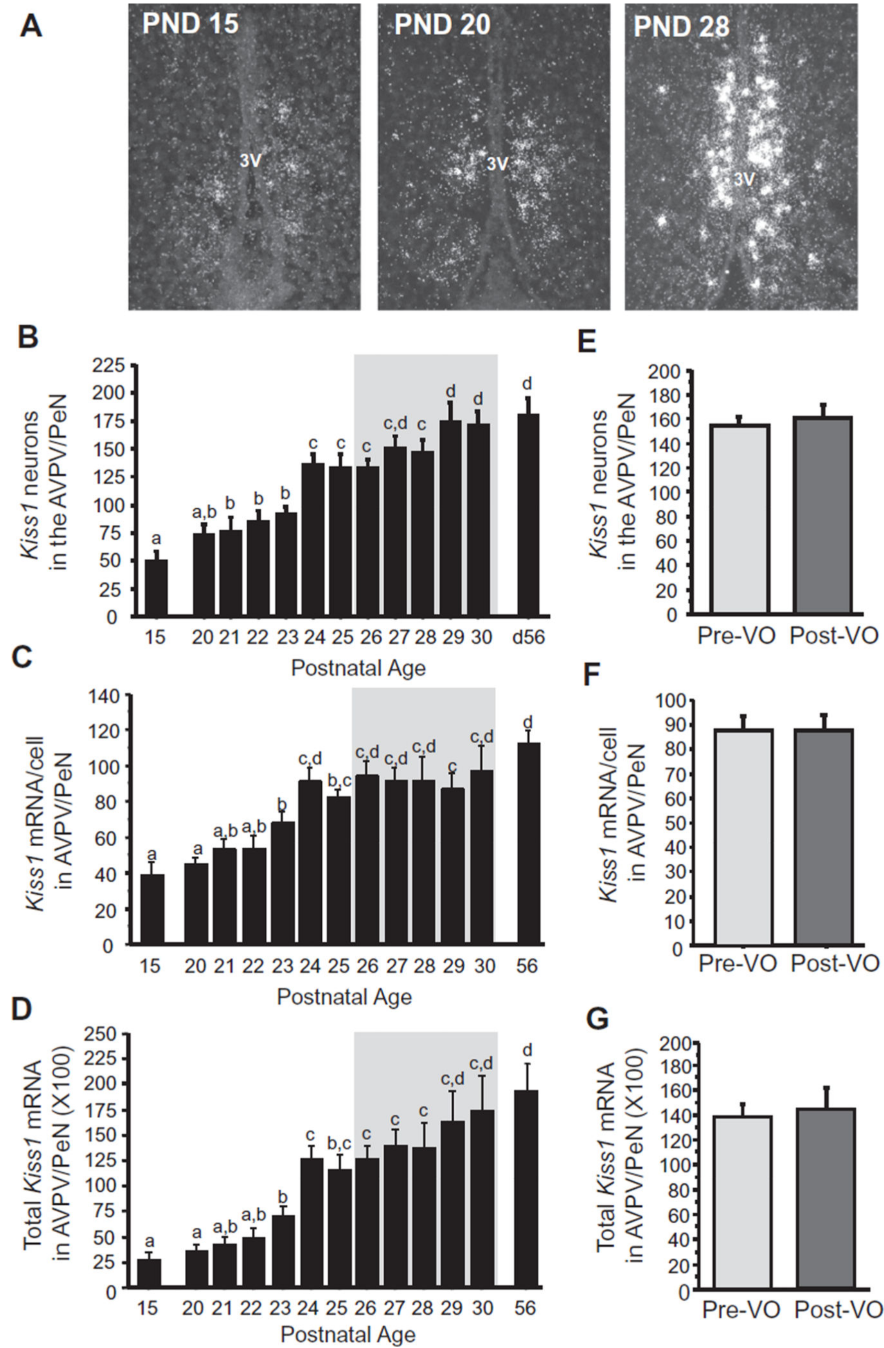

Fig. 2.

Kiss1 expression in the AVPV/PeN of female prepubertal and pubertal mice. (A) Representative images of *Kiss1* expression, determined by ISH, in the AVPV/PeN of female mice. 3V, third ventricle. (B) Mean numbers of *Kiss1* neurons in the AVPV/PeN, (C) mean relative *Kiss1* mRNA content per neuron in the AVPV/PeN, and (D) mean relative total *Kiss1* mRNA in the AVPV/PeN of female mice between PND 15 and PND 30, with adult diestrus female (PND 56) shown for comparison. The gray shading denotes the period when VO, an external marker of puberty, was observed (PND 26–30). (E–G) *Kiss1* cell numbers,

mRNA per cell, and total mRNA in the AVPV/PeN in female mice sacrificed just during the VO period (denoted by the gray shaded area in the other graphs) and analyzed based on status of VO, independent of age. Different letters denote significantly different from each other.

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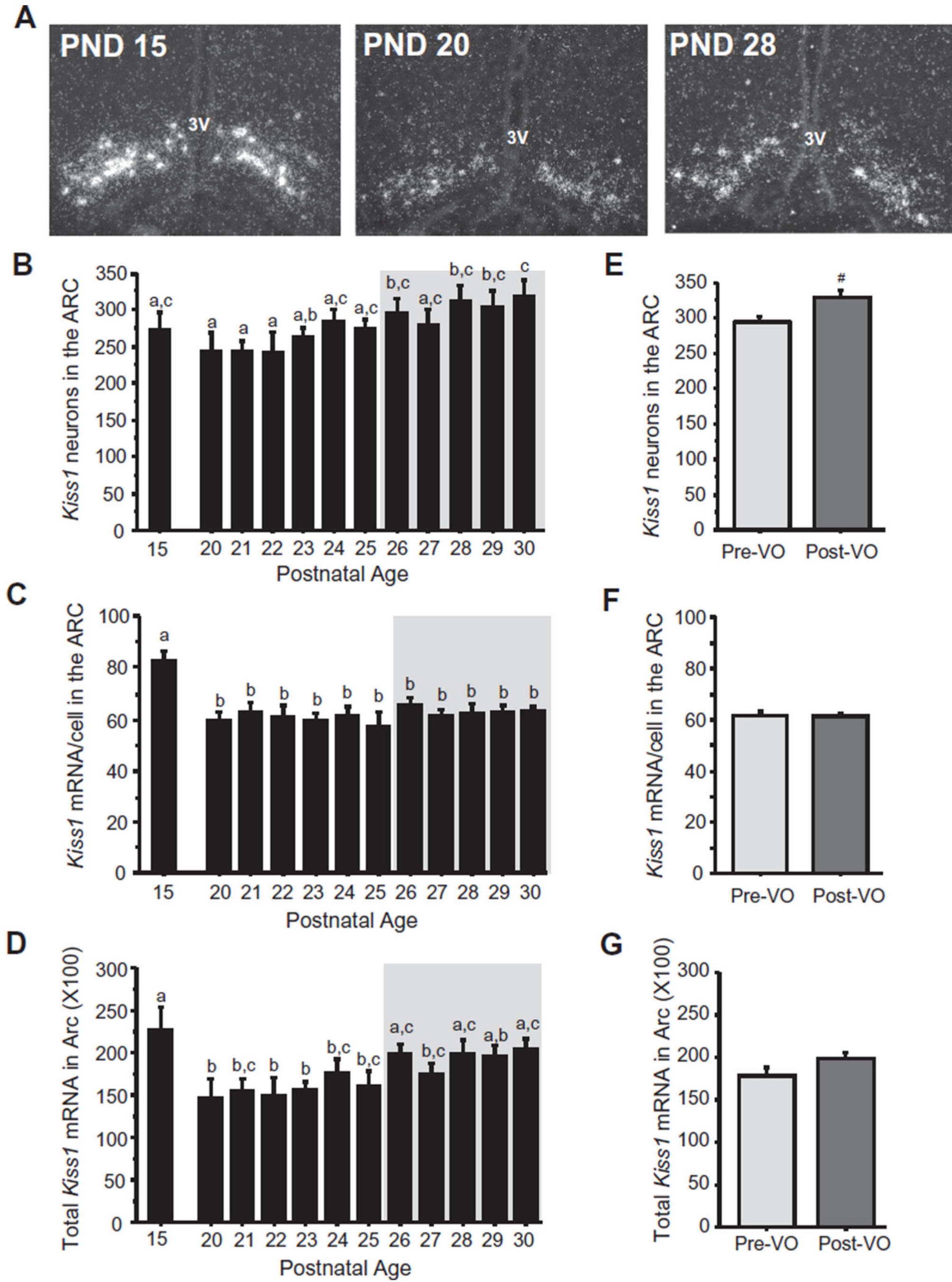

Fig. 3.

Kiss1 expression in the ARC of female prepubertal and pubertal mice. (A) Representative images of *Kiss1* expression, determined by ISH, in the ARC of female mice. 3V, third ventricle. (B) Mean numbers of ARC *Kiss1* neurons, (C) mean relative *Kiss1* mRNA content per neuron in the ARC, and (D) mean relative total *Kiss1* mRNA in the ARC of female mice between PND 15 and PND 30, with adult diestrus female (PND 56) shown for comparison. The gray shading denotes the period when VO was observed (PND 26–30). Different letters denote significantly different from each other. (E–G) *Kiss1* cell numbers, mRNA per cell,

and total mRNA in the ARC in female mice sacrificed just during the VO period (denoted by the gray shaded area in the other graphs) and analyzed based on VO status, independent of age. #, non-significant trend ($p = 0.054$).

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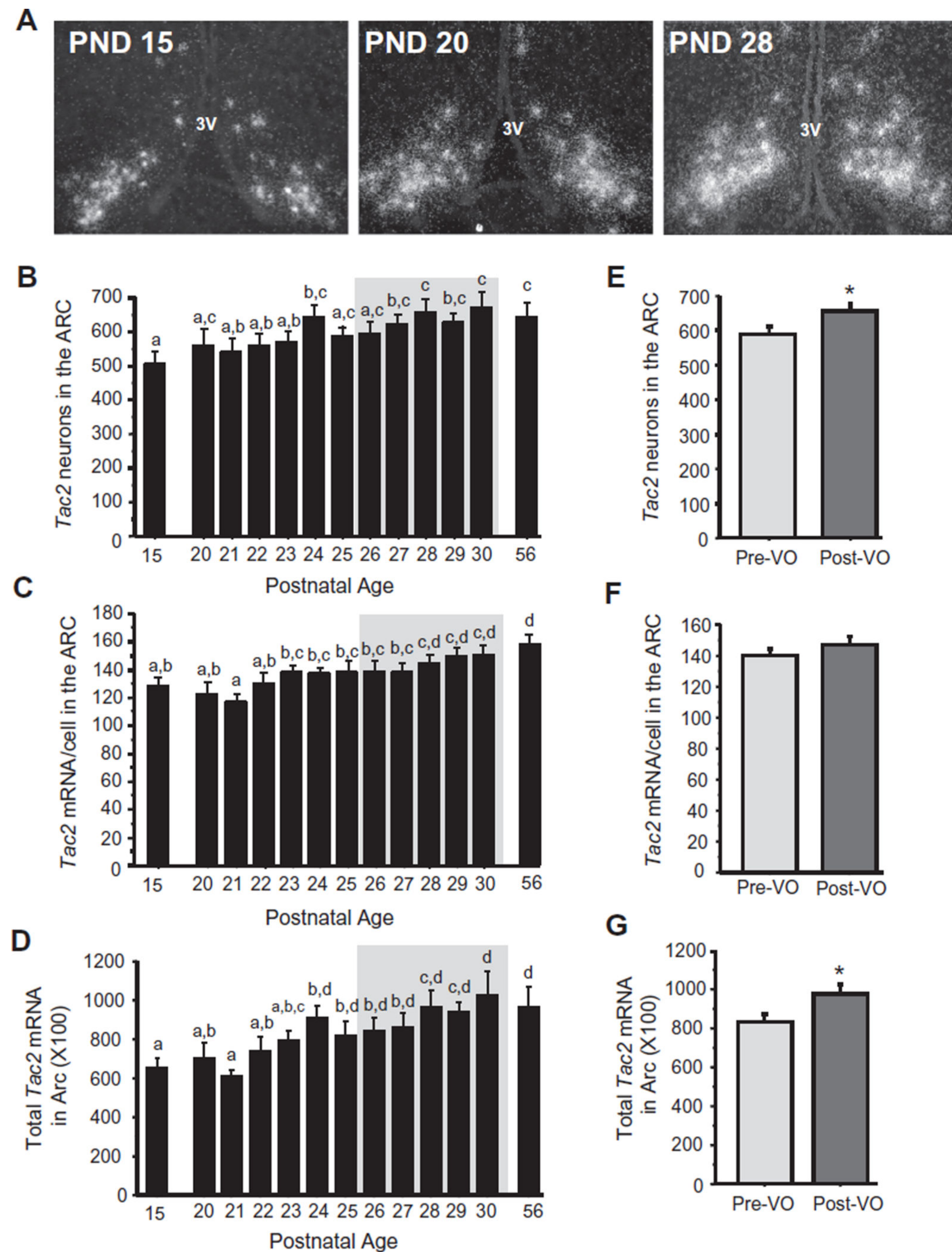

Fig. 4.

Tac2 expression in the ARC of female prepubertal and pubertal mice. (A) Representative images of *Tac2* expression, determined by ISH, in the ARC of female mice. 3V, third ventricle. (B) Mean numbers of ARC *Tac2* neurons, (C) mean relative *Tac2* mRNA content per neuron in the ARC, and (D) mean relative total *Tac2* mRNA in the ARC of female mice between PND 15 and PND 30, with adult diestrus female (PND 56) shown for comparison. The gray shading denotes the period when VO was observed (PND 26–30). (E–G) *Tac2* cell numbers, mRNA per cell, and total mRNA in the ARC of female mice sacrificed during the

VO period and analyzed based on the status of VO, independent of age. Different letters denote significantly different from each other. *, significantly different from pre-VO status.

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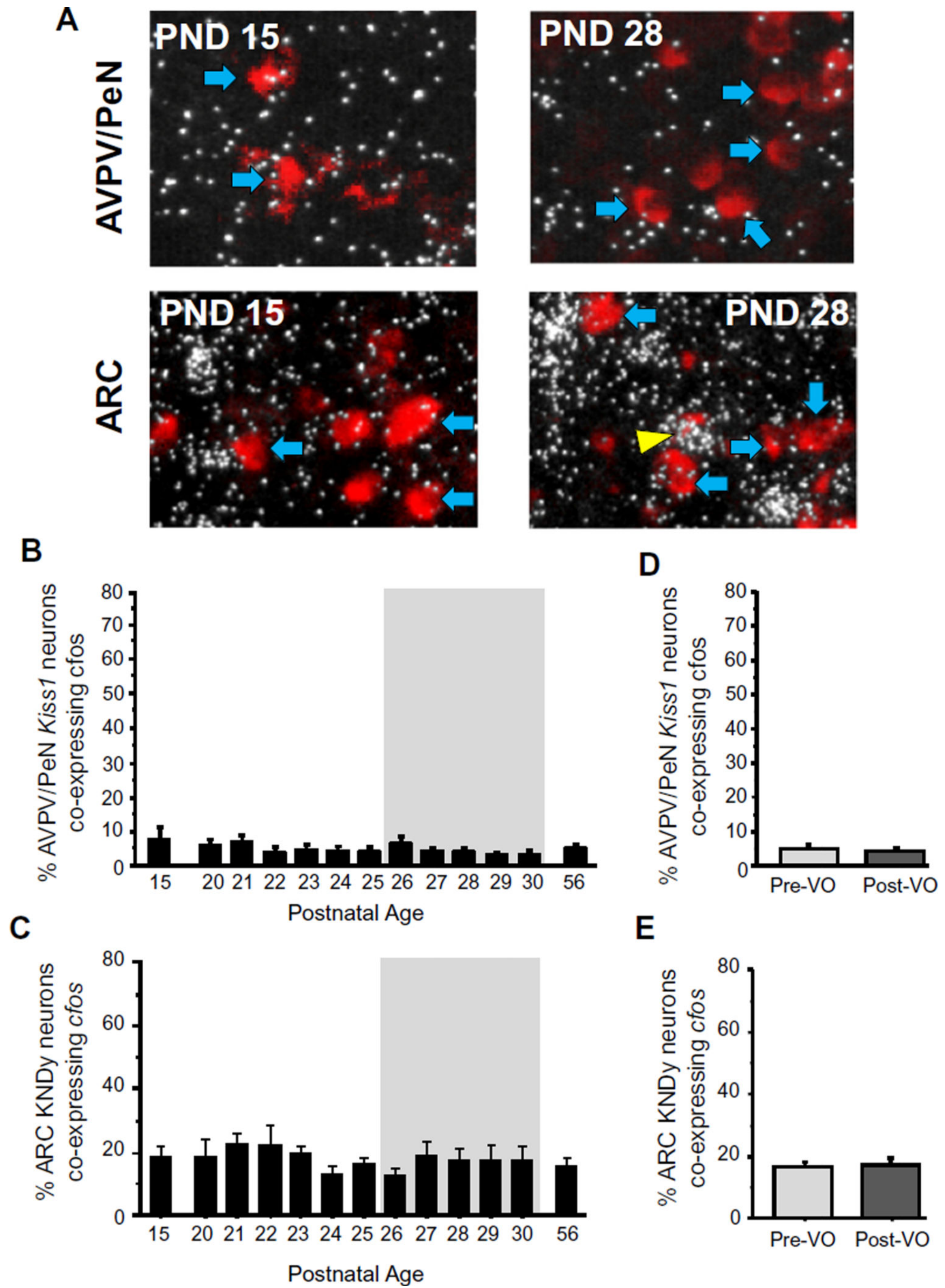

Fig. 5.

Kiss1 neuronal activation during female peri-pubertal development. (A) Representative images of *cfos* mRNA expression (silver grains) in *Kiss1* neurons (red fluorescence) in the AVPV/PeN and ARC of peri-pubertal female mice. Yellow arrowheads denote examples of *Kiss1-cfos* co-expression. Blue arrowheads denote example *Kiss1* cells without *cfos*. (B and C) Mean percent of AVPV/PeN and ARC *Kiss1* neurons expressing *cfos* in female mice between PND 15 and PND 30, and on PND 56 (diestrus adult female controls). The gray shading denotes the period when VO was observed. (D and E) *Kiss1-cfos* coexpression in

female mice from the VO period analyzed based on each female's status of VO. Different letters denote significantly different from each other. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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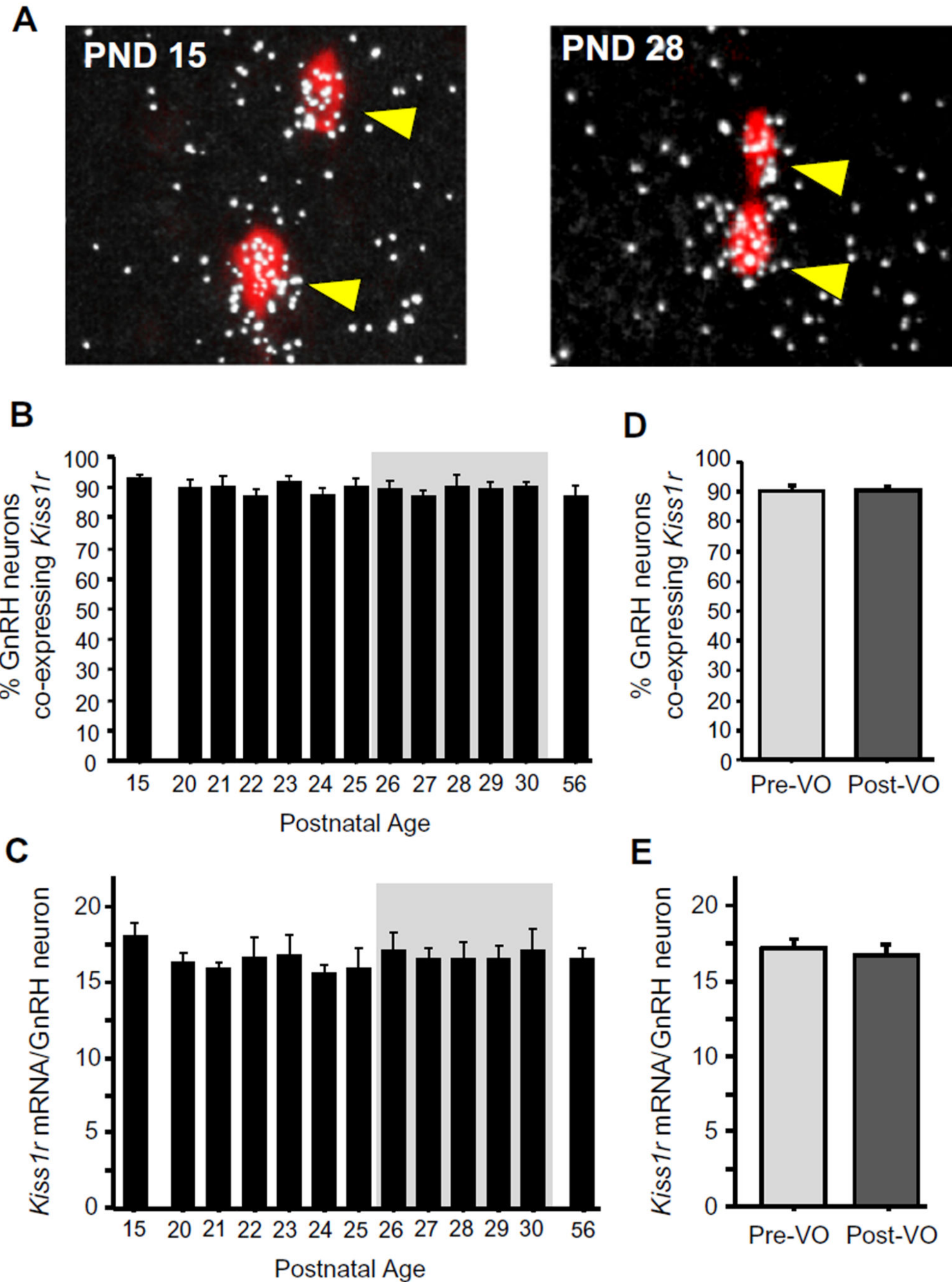

Fig. 6.

Kisspeptin receptor levels in GnRH neurons during female peri-pubertal development. (A) Representative images of *Kiss1r* mRNA expression (silver grains) in *Gnrh* neurons (red fluorescence) in peri-pubertal female mice. (B) Mean percent of *Gnrh* neurons expressing *Kiss1r* in female mice between PND 15 and PND 30, and on PND 56 (diestrus adult female controls). (C) Mean relative levels of *Kiss1r* mRNA per GnRH neuron across the pubertal transition. (D and E) *Gnrh-Kiss1r* coexpression in female mice from the VO period analyzed based on each female's status of VO. Different letters denote significantly different from

each other. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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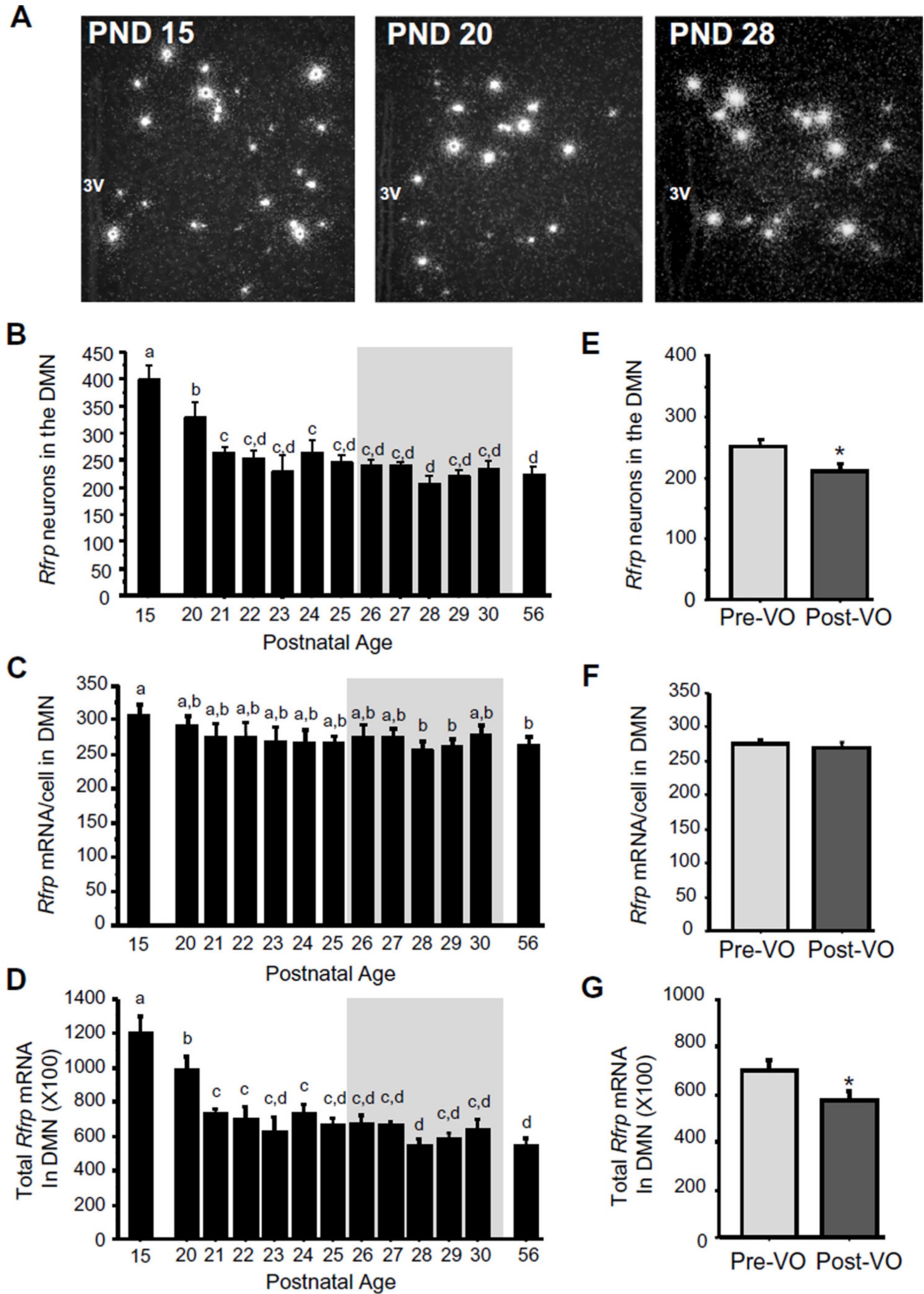

Fig. 7.

Rfrp expression in the DMN of female peri-pubertal mice. (A) Representative images of *Rfrp* expression, determined by ISH, in the DMN of female mice. 3V, third ventricle. (B) Mean numbers of *Rfrp* neurons, (C) mean relative *Rfrp* mRNA content per neuron, and (D) mean relative total *Rfrp* mRNA in of female mice between PND 15 and PND 30, with adult diestrus female (PND 56) shown for comparison. The gray shading denotes the period when VO was observed (PND26–30). (E–G) *Rfrp* cell numbers, mRNA per cell, and total mRNA in the DMN in female mice from the VO period (shaded gray area in other graphs) and

analyzed based on each female's VO status, independent of age. Different letters denote significantly different from each other. *, significantly different from pre-VO status.

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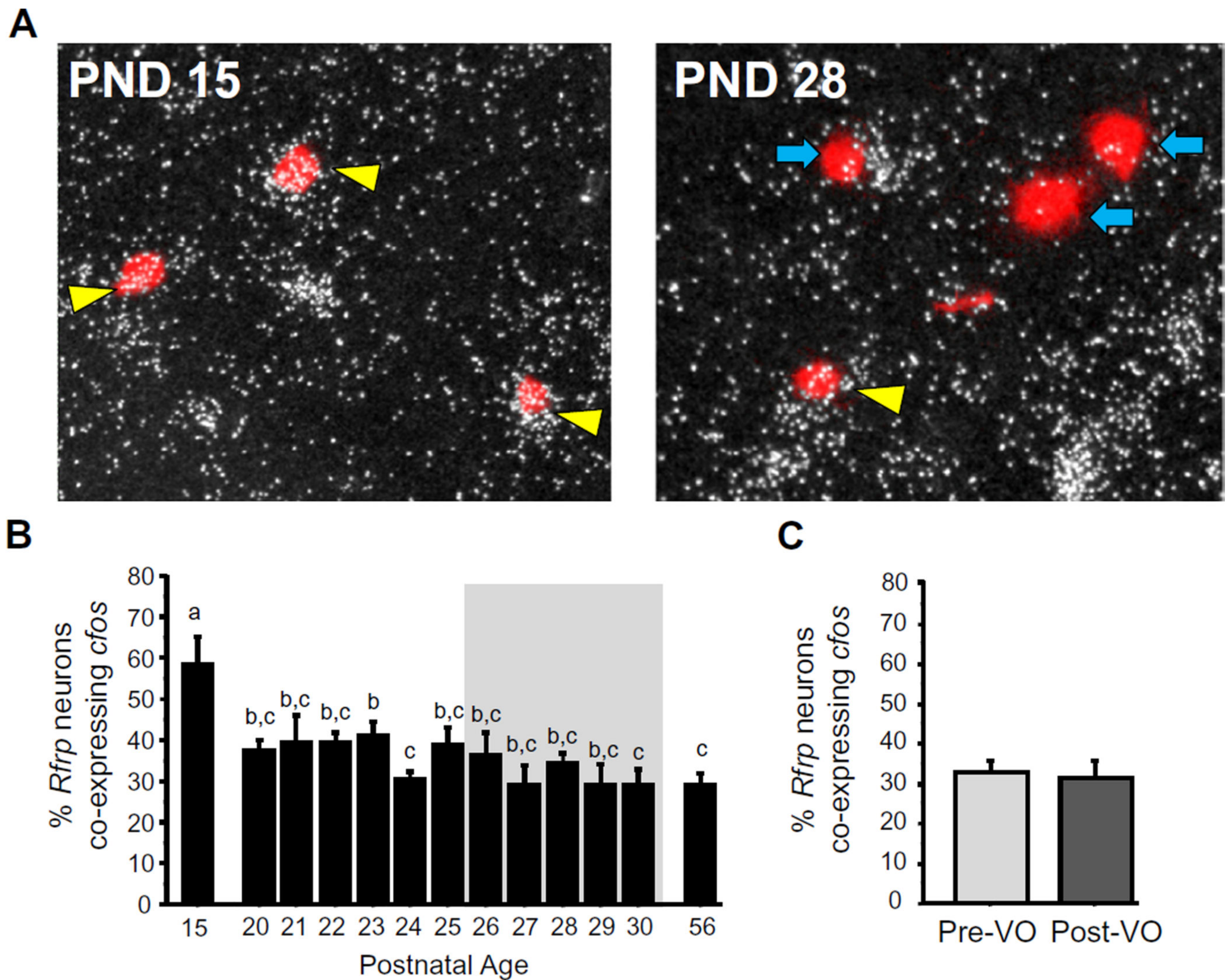

Fig. 8.

RFRP-3 neuronal activation during female peri-pubertal development. (A) Representative images of *cfos* mRNA expression (silver grains) in *Rfrp* neurons (red fluorescence) in the DMN of peri-pubertal female mice. Yellow arrowheads denote examples of *Rfrp-cfos* co-expression. Blue arrowheads denote example *Rfrp* cells without *cfos*. (B) Mean percent of *Rfrp* neurons expressing *cfos* in female mice between PND 15 and PND 30, and on PND 56 (diestrus adult female controls). The gray shading denotes the period when VO was observed (PND26–30). (C) *Rfrp-cfos* coexpression in female mice from the VO period analyzed based on each female's status of VO, independent of age. Different letters denote significantly different from each other. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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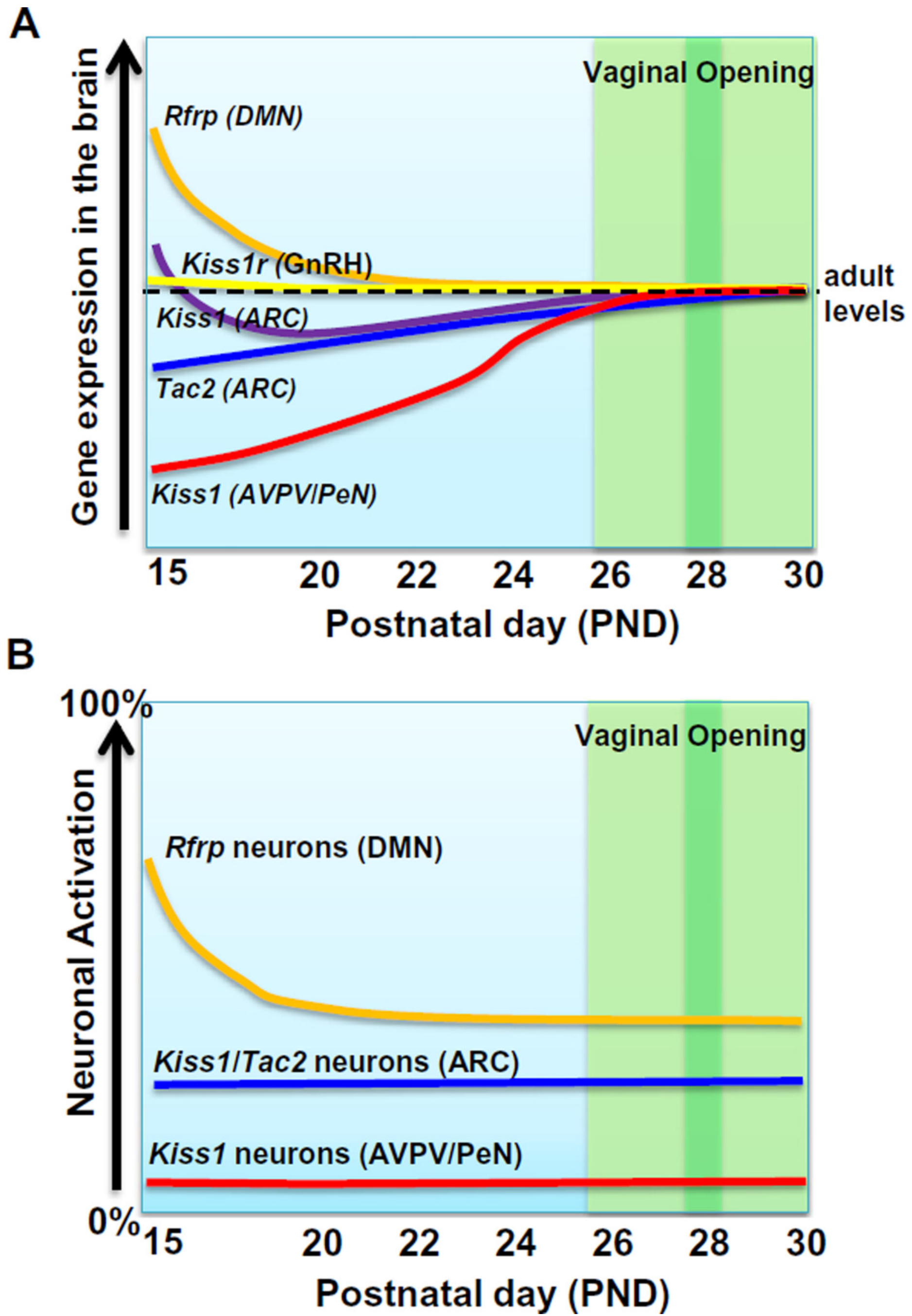

Fig. 9.

Cartoon schematic summarizing the various changes in reproductive gene expression and neuronal activation during the pubertal transition in female mice. (A) Summary of the changes in neural gene expression in reproductive genes during the pubertal transition in female mice. Levels are plotted relative to typical adult levels, which are designated by the horizontal black dotted line. (B) Summary of the changes in neuronal activation in reproductive circuits (kisspeptin and RFRP-3 neuronal populations) during the pubertal transition. Relative levels are plotted as 0–100% of each neuronal population showing

activation. In both (A) and (B), the green shaded area designates the period when vaginal opening (VO) is observed, with the darker green bar denoting the mean age of VO. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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Table 1

Correlation matrix showing correlation analysis of neural reproductive gene expression and neuronal activation during the pubertal period of female mice. Values shown are Pearson correlation coefficients, which can range from −1.0 to 1.0, with positive and negative values reflecting a positive correlation or negative correlation, respectively. Values in bold (positive) or italic (negative) are statistically significant ($p < 0.05$); non-bold, non-italic values are not statistically significant.

	AVPV Kiss1 cells	**AVPV Kiss1 total mRNA**	**ARC Kiss1 cells**	**ARC Kiss1 total mRNA**	**ARC Tac2 cells**	**ARC Tac2 total mRNA**	**Rfrp cells**	**Rfrp total mRNA**	**AVPV neural activat.**	**ARC neural activat.**	**Rfrp neural activat.**
AVPV Kiss1 cells		**0.884**	**0.422**	0.206	**0.399**	**0.469**	*−0.521*	*−0.560*	−0.200	0.228	*−0.383*
AVPV Kiss1 total mRNA	**0.884**		**0.296**	0.105	**0.329**	**0.410**	*−0.455*	*−0.495*	−0.204	*−0.270*	*−0.301*
ARC Kiss1 cells	**0.422**	**0.296**		**0.782**	**0.537**	**0.546**	−0.058	−0.067	−0.090	−0.120	*−0.364*
ARC Kiss1 total mRNA	0.206	0.105	**0.782**		**0.301**	**0.328**	**0.263**	**0.246**	−0.030	−0.051	−0.191
ARC Tac2 cells	**0.399**	**0.329**	**0.537**	**0.301**		**0.898**	*−0.211*	*−0.243*	−0.132	−0.107	*−0.354*
ARC Tac2 total mRNA	**0.469**	**0.410**	**0.546**	**0.328**	**0.898**		*−0.241*	*−0.277*	−0.224	−0.019	*−0.322*
Rfrp cells	*−0.521*	*−0.455*	−0.058	**0.263**	*−0.211*	−0.241		**0.883**	0.114	0.123	**0.379**
Rfrp total mRNA	*−0.560*	*−0.495*	−0.067	**0.246**	*−0.243*	*−0.277*	**0.883**		0.050	0.176	**0.471**
AVPV Kiss1 neural activat.	−0.200	−0.204	−0.090	−0.030	−0.132	−0.224	0.114	0.050		0.041	0.006
ARC Kiss1 neural activat.	0.228	*−0.270*	−0.120	−0.051	−0.107	−0.019	0.123	0.176	0.041		0.091
Rfrp neural activat.	*−0.383*	*−0.364*	*−0.364*	−0.191	*−0.354*	*−0.322*	**0.379**	**0.471**	0.006	0.091	