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RESEARCH ARTICLE

Selective depletion of kisspeptin neurons in the hypothalamic arcuate nucleus in early juvenile life reduces pubertal LH secretion and delays puberty onset in mice

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Abstract

Puberty is the critical developmental transition to reproductive capability driven by the activation of gonadotropin-releasing hormone (GnRH) neurons. The complex neural mechanisms underlying pubertal activation of GnRH secretion still remain unknown, yet likely include kisspeptin neurons. However, kisspeptin neurons reside in several hypothalamic areas and the specific kisspeptin population timing pubertal onset remains undetermined. To investigate this, we strategically capitalized on the differential ontological expression of the *Kiss1* gene in different hypothalamic nuclei to selectively ablate just arcuate kisspeptin neurons (aka KNDy neurons) during the early juvenile period, well before puberty, while sparing RP3V kisspeptin neurons. Both male and female transgenic mice with a majority of their KNDy neurons ablated (KNDy^{ABL}) by diphtheria toxin treatment in juvenile life demonstrated significantly delayed puberty onset and lower peripubertal LH secretion than controls. In adulthood, KNDy^{ABL} mice demonstrated normal in vivo LH pulse frequency with lower basal and peak LH levels, suggesting that only a small subset of KNDy neurons is sufficient for normal GnRH pulse timing but more KNDy cells are needed to secrete normal LH concentrations. Unlike prior KNDy ablation studies in rats, there was no alteration in the occurrence or magnitude of estradiol-induced LH surges in KNDy^{ABL} female mice, indicating that a complete KNDy neuronal population is not essential for normal LH surge generation. This study teases apart the contributions of different kisspeptin neural populations to the control of puberty onset, demonstrating that a majority of KNDy neurons in the arcuate nucleus are necessary for the proper timing of puberty in both sexes.

Abbreviations: 3V, 3rd ventricle; ARC, arcuate nucleus; BW, body weight; CON, controls; DT, diphtheria toxin; DTR, diphtheria toxin receptor; E2, estradiol; FE, first estrus; GDX, gonadectomized; GnRH, gonadotropin releasing hormone; ISH, in situ hybridization; KNDy, Kisspeptin, NKB, and dynorphin; KNDy^{ABL}, ablated KNDy neurons; LH, luteinizing hormone; NKB, neurokinin B; PS, preputial separation; RP3V, rostral periventricular nucleus of the 3rd ventricle; VO, vaginal opening.

KEYWORDS
GnRH, hypothalamus, Kiss1, kisspeptin, LH pulses, LH surge, luteinizing hormone, puberty, reproduction, sexual maturation

1 | INTRODUCTION

A neuroendocrine hallmark of the onset of puberty is a developmental increase in the brain's pulsatile secretion of gonadotropin-releasing hormone (GnRH) which triggers a corresponding increase in the downstream pituitary secretion of luteinizing hormone (LH).[1–5] The complex neural mechanisms controlling the timely developmental activation of GnRH and LH secretion during the initiation of puberty are still not fully understood, but likely involve neurons synthesizing the neuropeptide kisspeptin.[1,2,6–8] Encoded by the *Kiss1* gene, kisspeptin directly stimulates GnRH neurons, evoking GnRH and downstream LH secretion.[8–11] The involvement of kisspeptin signaling in puberty is supported by seminal findings in humans and rodents that mutations in either *Kiss1* or its receptor (*Kiss1r*) result in hypogonadism and impaired puberty onset.[12–16] Likewise, blocking endogenous kisspeptin signaling with a kisspeptin receptor antagonist delays puberty onset in rats.[17] Two major populations of kisspeptin neurons reside in the hypothalamus, one in the arcuate nucleus (ARC) and another more rostrally in the preoptic area, which in rodents is the rostral periventricular nucleus of the 3rd ventricle (RP3V; also called the AVPV/PeN). ARC kisspeptin neurons co-express neurokinin B (NKB, encoded by *Tac2*) and dynorphin, and are often termed KNDy neurons.[18–20] Like kisspeptin, NKB signaling is stimulatory to the reproductive axis and critical for normal puberty onset.[21–26] Recent studies in mice suggest a key role for KNDy neurons in the neural GnRH pulse generator mechanism that governs pulsatile GnRH and LH secretion.[9,11,27,28] In contrast, RP3V *Kiss1* neurons are not implicated in pulsatile GnRH and LH secretion but instead are thought to drive the estrogen-induced preovulatory LH surge that occurs in adult females (reviewed in Refs.[29–31]).

In rodents, there is a developmental increase in *Kiss1* gene expression in both the ARC and RP3V nuclei during the peri-pubertal period,[32,33] but importantly, the ARC and RP3V kisspeptin cell populations have different ontological patterns during earlier development periods (reviewed in Semaan et al.[6]). In both mice and rats, ARC neurons first start expressing high levels of *Kiss1* mRNA (or kisspeptin protein) a few days before birth[34–37] and the number of detectable ARC *Kiss1* neurons is already high and near adult levels at infantile and early juvenile ages, well before puberty begins.[7,32,38–40] Following these high *Kiss1* expression levels in early juvenile life, ARC *Kiss1* levels of mice and rats transiently decrease during the late juvenile and pre-pubertal period before slowly increasing again back to high levels during the pubertal stage.[32,33,38,41] Similarly, ARC *Tac2* cell numbers and expression levels in mice slowly increase during the peri-pubertal period.[32,33,42] In contrast to the ARC population, RP3V kisspeptin neurons are not yet present before birth or during the neonatal, infantile, or early juvenile stages.[6,7,35,43,44] In both mice and rats, a few RP3V *Kiss1* neurons are first detected around the end of the 2nd week of postnatal life (~postnatal day [PND] 10–14) but only at very small numbers (≤5% of adult cell counts).[40,44–47] RP3V *Kiss1* cell numbers first start to rise dramatically a week or two later near the end of the 3rd postnatal week, establishing high levels by the end of the 4th week, around puberty onset (PND 28–30).[7,32,41,45,48] While both RP3V kisspeptin and ARC kisspeptin/NKB cell populations show peri-pubertal increases, it is not currently known if such increases contribute to the pubertal rise in LH secretion or the timing of puberty. In fact, the specific kisspeptin cell population(s) responsible for driving puberty onset still remains to be experimentally demonstrated.

Several prior studies in rodents employed cell ablation techniques to permanently remove kisspeptin neurons and study in vivo function.[49–53] However, the design of those previous ablation studies was not able to tease apart the role of select kisspeptin cell populations in the pubertal process. The earliest study, performed in female mice, ablated *all* kisspeptin neurons throughout life, beginning at prenatal ages, and therefore could not determine the contribution of specific kisspeptin populations in the various reproductive assessments, including puberty, nor could it rule out the likelihood of developmental compensation owing to the very early (prenatal) age of ablation.[49] By contrast, several studies in rats partially ablated (~70%) just the ARC kisspeptin (i.e., KNDy) cell population using neurokinin 3 receptor agonists conjugated to saporin, but such KNDy ablation was only induced in adulthood after puberty was fully completed.[50–53] Those adult rats studies also only studied ablation effects in females and did not include males.[50–53] Thus, no prior study has selectively ablated just a single kisspeptin cell population early enough in postnatal development in either sex to permit subsequent analysis of the timing of puberty onset, which in female mice typically occurs ~PND 27–30 and in males ~PND 29–32 (as determined with common anatomical markers, vaginal opening, and preputial separation, respectively).

The present study had one primary goal and two secondary goals. Our primary goal was to tease apart the possible role of ARC versus RP3V kisspeptin neuron populations in puberty onset by selectively ablating just one cell population in early postnatal development while sparing the other population. Because a neuroendocrine hallmark of puberty onset is the developmental increase in pulsatile GnRH secretion,[3,54] we hypothesized that the ARC kisspeptin population (i.e., KNDy neurons, which comprise the GnRH pulse generator[11,55,56]) drives the developmental initiation of puberty. To test this, we selectively ablated just ARC kisspeptin neurons in early juvenile development, multiple weeks before normal puberty onset occurs and without altering the RP3V kisspeptin population, and then studied puberty timing and pubertal LH levels in both sexes. While analysis of puberty onset was our main focus, given the proposed importance of KNDy neurons in controlling LH pulses,[9,28,56] a secondary goal was to assess whether the juvenile ablation of most KNDy neurons also compromises adult in vivo LH pulse secretion patterns, which was not analyzed in prior study that ablated KNDy neurons.[50–52] Finally, given several reports of enhanced magnitude LH surges in adult female rats with partial ablation of KNDy neurons,[51–53] we also determined if estrogen-induced LH surges were altered or not in adult female mice that had sustained KNDy neuron depletion earlier as juveniles.

2 | MATERIALS AND METHODS

2.1 | Animals

Transgenic mice expressing inducible diphtheria toxin receptor (DTR) exclusively in kisspeptin cells were generated by crossing *Kiss1* Cre female mice (provided by Dr. Carol Elias) with males from a DTR knock-in line (Stock No. 007900; Jackson Labs, USA) that has a floxed stop codon upstream of a DTR allele. Mice normally do not express DTR in any cells; however, in transgenic *Kiss1* Cre+/iDTR mice, Cre recombinase expressed in kisspeptin cells will remove the stop codon from the DTR allele, allowing for constitutive expression of DTR in just those kisspeptin cells. Exogenous diphtheria toxin (DT) treatment can bind DTR to induce cell death, thereby selectively ablating cells that express DTR. Our breeding backcrosses ultimately generated *Kiss1* $Cre^{+}/iDTR^{fl/fl}$ offspring (expressing DTR only in kisspeptin cells) along with *Kiss1* $Cre^{-}/iDTR^{fl/fl}$ control littermates. Because these control mice lack Cre recombinase, they do not express DTR in any cells and are not susceptible to DT treatment.

For examination and confirmation of the normal number of *Kiss1* neurons in the RP3V and ARC of juvenile mice at PND 12 and pubertal mice at PND 28, transgenic mice expressing Cre-dependent tdtomato (red fluorescence) in neurons expressing the *Kiss1* gene were used (*Kiss1* Cre x $Rosa^{todtomato}$; "$Kiss1^{tdtom}$"). In these mice, when the *Kiss1* gene is first expressed, it drives Cre recombinase-mediated excision of a stop codon in front of the tdtomato allele, permitting constitutive tdtomato expression thereafter, regardless of future *Kiss1* gene expression status. This allows for permanent "marking" of kisspeptin-expressing neurons following their first expression of the *Kiss1* gene.

In all cases, the day of birth was designated as PND 1 and all animals were weaned at PND 21 and group housed (2–4 per cage), with ad libitum access to food and water. The mouse room was maintained on a 12 h dark:12 h light cycle with lights off at 18:00 h. All animal experiments were approved and followed the guidelines set by the IACUC at the University of California San Diego.

2.2 | Quantification of *Kiss1* cell numbers in the ARC and RP3V of juvenile mice

To first confirm differential ontological patterns of *Kiss1* expression in RP3V and ARC neurons, we used transgenic mice permanently expressing tdtomato red fluorescence in neurons expressing the *Kiss1* gene ($Kiss1^{tdtom}$). Heterozygous $Kiss1^{tdtom}$ mice were sacrificed on either PND 12 (juvenile) or PND 28 (pubertal) and their brains were collected into cold 4% paraformaldehyde. Fixed brains were further soaked overnight in 4% paraformaldehyde at 4°C and were then transferred to 30% sucrose at 4°C until the brains had sunk. Fixed brains were later cut on a cryostat into 5 sets of 20 μm sections and stored at −80°C. For each animal, one set of brain sections was analyzed under fluorescent microscopy and the number of kisspeptin neurons (red fluorescence) was counted in both the RP3V and ARC brain regions.

2.3 | Strategy for the selective ablation of ARC *Kiss1* neurons before puberty

Previous studies ablating kisspeptin neurons either ablated *all* kisspeptin neuron populations or only ablated the ARC kisspeptin population in adulthood.[49–51] To selectively ablate just ARC kisspeptin neurons well before the pubertal period while sparing RP3V kisspeptin neurons, we took advantage of the different developmental ontogenies of *Kiss1* expression in the two kisspeptin populations (summarized in Figure 1A). In juvenile *Kiss1* $Cre^{+}/iDTR^{fl/fl}$ mice, most ARC *Kiss1* neurons will already express DTR and be susceptible to significant

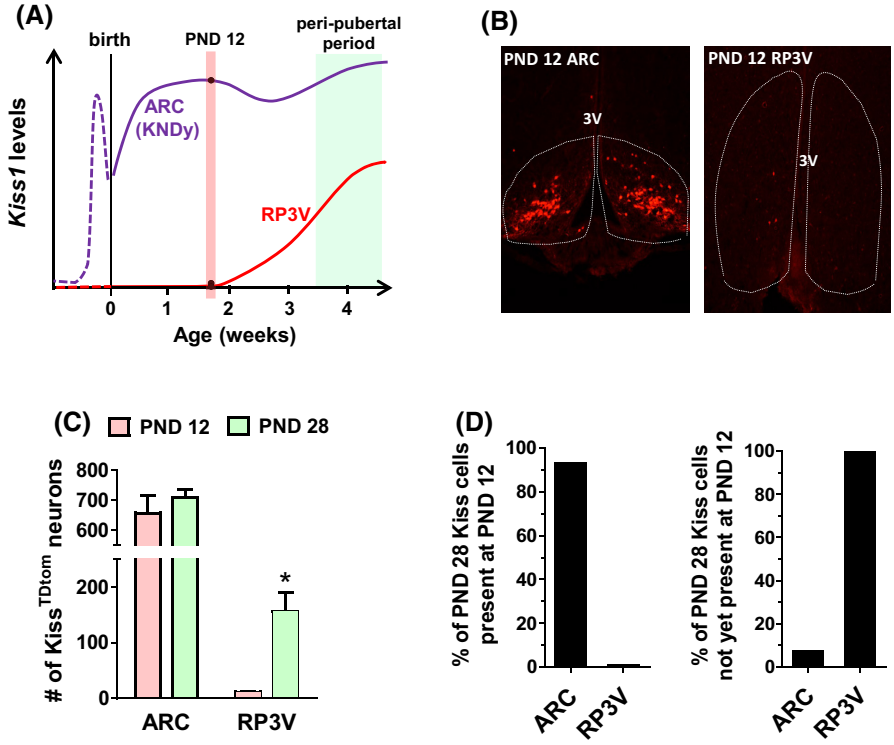

FIGURE 1 Differential kisspeptin cell numbers in juvenile development. (A) Schematic summarizing previously-reported data (reviewed in Semaan et al.[6]) on *Kiss1* or kisspeptin cell numbers in the ARC and RP3V in rodents during prenatal, juvenile, and pubertal periods, demonstrating that many ARC cells are already present at neonatal and juvenile ages compared to the RP3V which lacks notable detectable kisspeptin cells in the juvenile period. The light red shaded region denotes the juvenile age PND 12 when DT was given in the present study and the green shaded region denotes the pubertal period. (B) Representative images of kisspeptin cells, represented by Cre-mediated tdtomato fluorescence, demonstrating many kisspeptin cells present in the ARC (*left*) but virtually no cells present in the RP3V (*right*) at the juvenile age of PND 12. 3V denotes the 3rd ventricle. (C) Quantification of kisspeptin-tdtomato cell number in the ARC and RP3V at PND 12 and PND 28 (juvenile and pubertal ages, respectively). *$p < .05$ versus PND 12. (D) Calculation of the % of PND 28 kisspeptin cells present at PND 12 (*left*) and the % of PND 28 kisspeptin cells *not* present at PND 12 (*right*), demonstrating that most PND 28 ARC kisspeptin cells are already present at PND 12, unlike RP3V kisspeptin cells which are virtually absent at PND 12 and not readily present until the later pubertal age.

ablation by acute DT treatment at this age; by contrast, minimal RP3V *Kiss1* expression has occurred at this age and the majority of the RP3V *Kiss1* population will not yet express DTR. We therefore injected DT (75 mL of 0.1 mg/mL) or vehicle (saline) intraperitoneally to juvenile *Kiss1* $Cre^{+}/iDTR^{fl/fl}$ males and females and their Cre-control littermates on PND 12 and PND 13, when DTR would be expressed primarily only in the ARC kisspeptin cell population (Figure 2A). *Kiss1* Cre+/$iDTR^{fl/fl}$ mice of each sex receiving juvenile DT treatment are henceforth referred to as "$KNDy^{ABL}$" (ABL = *abl*ation induced in juvenile life) males and females. For controls, we used both *Kiss1* $Cre^{+}/iDTR^{fl/fl}$ littermates given vehicle instead of DT as well as *Kiss1* $Cre^{-}/iDTR^{fl/fl}$ littermates given DT (collectively termed "controls" or CON). There were no statistical group differences in any measure between these two control cohorts, and we therefore combined them into a single "control" group for each sex to maximize statistical power.

2.4 | ISH for *Kiss1* mRNA expression

To validate the selective ablation of ARC *Kiss1* neurons, in situ hybridization (ISH) for *Kiss1* gene expression was performed in brain slices containing the entire ARC region from adult gonadectomized (GDX) male and female $KNDy^{ABL}$ mice (previously given DT just in juvenile life, as described above) and control littermates. GDX mice were used to remove endogenous gonadal steroid feedback and increase *Kiss1* expression in the ARC, thereby enhancing the detection of any surviving *Kiss1* neurons. Fresh frozen brains collected in adulthood were sectioned on a cryostat at 20 mM into 5 alternating sets, mounted onto Superfrost Plus slides, and stored at −80°C. One set of brain sections containing the entire rostral to the caudal extent of the ARC was assayed for single-label fluorescent ISH to determine the number of *Kiss1* cells present, using our lab's standard ISH protocol and a well-validated digoxygenin-labeled

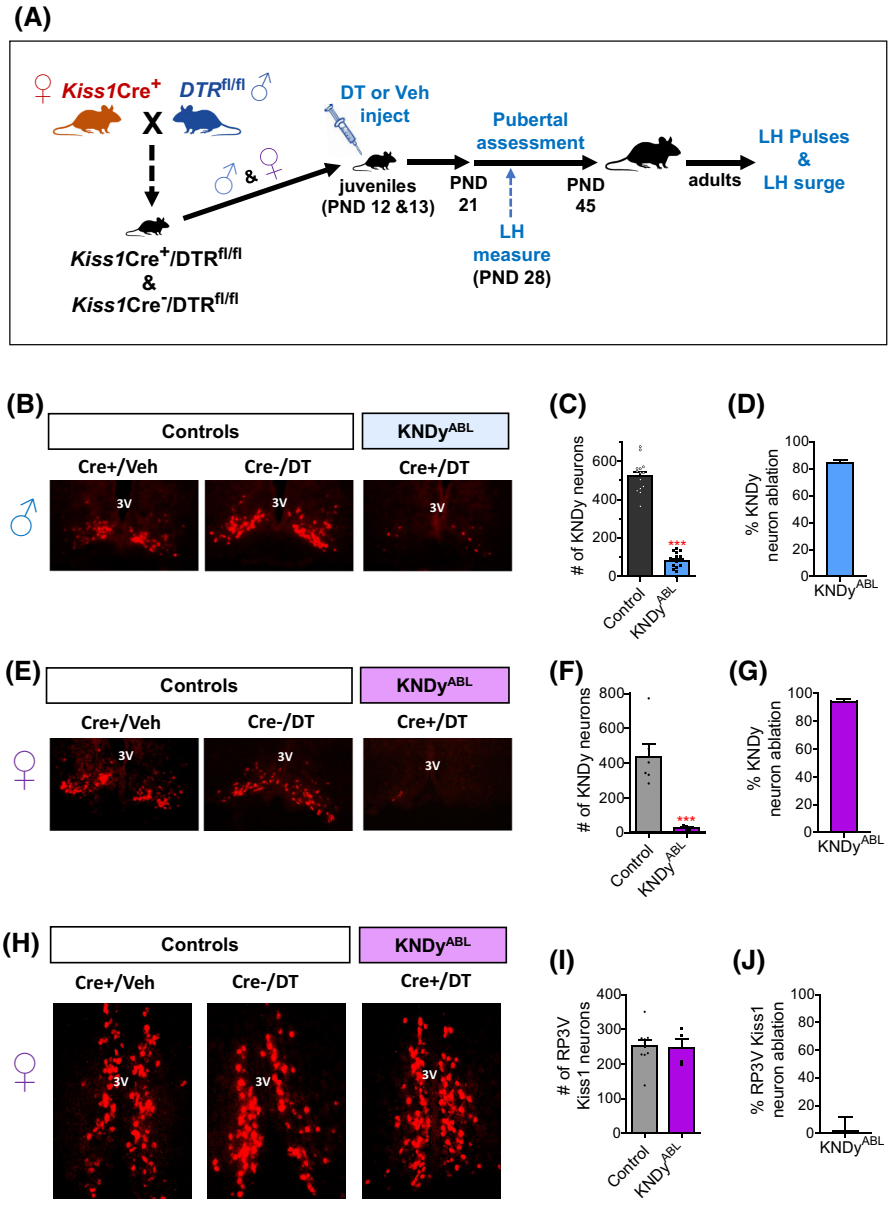

FIGURE 2 Experimental strategy and validation of selective ARC *Kiss1* (KNDy) neuron ablation induced in juvenile life. (A) Schematic representation of the experimental design to selectively ablate KNDy neurons based on the differential developmental onset of *Kiss1* expression in different hypothalamic populations. *Kiss1* Cre mice were crossed with DTR^{flox} mice to generate *Kiss1* Cre+/DTR^{flox} mice or Cre-/DTR^{flox} controls. DT treatment or vehicle was injected acutely in the juvenile period on PND 12 and PND 13 to induce kisspeptin cell death in Cre+ mice; in mice at these juvenile ages, *Kiss1* (and hence, DTR) has only been actively expressed at high levels in the ARC but *not yet* in the RP3V, permitting selective ablation of just ARC kisspeptin neurons at that age. Pubertal assessments and BWs were later determined from PND 21 to PND 45, and LH was measured in one-off samples taken in the peripubertal period on PND 28 and in adulthood. Adult mice sustaining KNDy neuron ablation in juvenile life were also examined for impairments in in vivo LH pulsatility and estrogen-induced LH surges. (B and E) Representative microscope images of ARC *Kiss1*-expressing neurons (red fluorescence) in adult gonadectomized males (B) and females (E), as detected with single-label ISH for *Kiss1* mRNA. 3V denotes the location of the 3rd ventricle. The mean number of ARC *Kiss1* neurons (C and F) and the degree of ARC *Kiss1* cell ablation (D and G) in male and female KNDY^{ABL} mice (blue or purple bars) is graphed versus control littermates (black or gray bars). $n = 14–16$/male group and $n = 4–6$/female group. (H) Representative microscope images of RP3V *Kiss1* neurons (red fluorescence) in adult ovariectomized females implanted with E_2. 3V denotes the 3rd ventricle. (I) The mean number of RP3V *Kiss1* neurons and (J) degree of RP3V *Kiss1* ablation in KNDY^{ABL} females (purple bars; $n = 4$) compared to control females (gray bars; $n = 9$). ***$p < .001$.

(DIG) *Kiss1* probe (1:500), as described previously.[57–61] For each animal, the number of red fluorescent DIG cells, representing *Kiss1* mRNA-expressing cells, was counted under a 20× objective using computer-assisted counting software (GRAINS, Dr. D. Clifton, University of Washington). The total number of ARC *Kiss1* cells

in individual KNDy^{ABL} male and female mice was compared to the mean number of total cells in control mice to determine ablation efficacy for each animal, expressed as a percentage (maximum ablation [no *Kiss1* cells left] = 100%, no ablation [all *Kiss1* cells remaining] = 0%). To confirm that juvenile DT treatment was specific to ARC kisspeptin neurons and did not reduce RP3V kisspeptin cells, a separate *Kiss1* ISH assay was similarly performed in brain slices containing the entire RP3V from adult OVX KNDy^{ABL} and control females treated with physiological estradiol (E_2) levels.

2.5 | Analysis of puberty onset and peri-pubertal LH levels

Puberty onset was determined in KNDy^{ABL} mice and control littermates for each sex. To avoid confounding effects of metabolic and body weight (BW) influences on puberty onset, which can occur in very small or very large litters,[62] only litters with 7–9 pups were used and occasional runts that were identified at the time of DT injection (PND 12 and 13) were excluded. All experimental males exhibited weaning weight (PND 21) between 8 and 10 g and all females weighed between 7 and 9 g at this age. Starting at PND 21, all mice were tracked daily for the emergence of common morphological pubertal markers: preputial separation (PS) in males and vaginal opening (VO) in females. BW was measured every 3 days for the entire pubertal assessment and on days when PS or VO were detected. After VO occurred in females, daily vaginal smears were taken for vaginal cytology to determine the day of first estrus (FE; a late pubertal marker signifying first ovulation), demonstrated by the presence of cornified cells. To assess peripubertal LH levels, a small tail-tip blood sample (5 mL) was collected on PND 28 from a subset of mice of each sex and mixed with 55 mL assay buffer. The resulting sample was stored at −20°C until assaying for LH by the University of Virginia Ligand Core via an ultra-sensitive mouse LH ELISA assay (see Hormone Assays below).

2.6 | Adult basal LH measurement and kisspeptin challenge

Because KNDy neurons govern pulsatile GnRH secretion and downstream LH secretion, we determined the effect of KNDy ablation sustained in juvenile life on basal LH secretion of gonad-intact mice in adulthood. To measure adult basal LH levels, a small tail-tip blood sample (4 mL) was collected from ~8-week-old control and KNDy^{ABL} male and female mice (in diestrus) in the morning. To confirm that any reductions in LH levels reflect an impairment at the level of KNDy neurons rather than impaired GnRH neuron or pituitary function, the basal LH sampling was followed by a kisspeptin challenge to interrogate the functionality of downstream GnRH and LH secretory cells. Adult mice of both sexes were injected i.p. with kisspeptin-10 (2 mg/kg, Cat. # 4243, Tocris Biosciences) immediately after the basal LH blood sample was collected. Ten minutes after the kisspeptin-10 injection, another 4 mL tail-tip blood sample was collected to measure the LH response to kisspeptin. All tail-tip blood samples were mixed with 56 mL of assay buffer, frozen at −20°C, and later assayed for LH levels using the ultra-sensitive mouse LH ELISA assay described below. For each animal, fold-response in LH secretion caused by kisspeptin-10 injection was calculated by comparing the post-kisspeptin LH value with the pre-kisspeptin (basal) starting LH value.

2.7 | In vivo LH pulse secretion in adulthood

Given compelling prior evidence that KNDy neurons govern LH pulse secretion,[9] we determined if selective KNDy depletion, initiated in juvenile life, impairs parameters of in vivo LH pulsatile secretion in adulthood. Adult KNDy^{ABL} males and littermate controls were gonadectomized (GDX) under isoflurane anesthesia at ~8 weeks of age to remove gonadal sex steroid negative feedback and enhance GnRH pulse generator output, a common paradigm to facilitate LH pulse detection in mice. Beginning 1 week later, mice were handled every day for 3 weeks to acclimatize to the serial tail-tip bleeding procedure. On the morning of the serial bleeding, the tip of the tail was snipped and 15 min later serial blood sampling began in which a tiny droplet of blood (3 mL) was collected from the tail tip with a sterile pipette tip, immediately mixed with 57 mL assay buffer, and stored on ice. Blood samples were collected from the tail every 6 min for an hour, as previously described.[63–65] After the serial bleeding was completed, mice were immediately euthanized and their brains were collected, frozen on dry ice, and stored at −80°C until processing for *Kiss1* ISH assay. LH levels in the serial samples were measured by the ultrasensitive murine LH ELISA performed by the University of Virginia Ligand Core, as in previous pulse studies.[61,64,66] LH pulse parameters, including pulse frequency, pulse amplitude, basal LH, and peak LH concentration, were calculated as in our previous LH pulse studies.[61,63,64,66] LH pulse parameters were analyzed with the PULSAR Otago software program using the following validated parameters outlined for GDX mice in the report by Porteous and colleagues[67]: smoothing 0.7, peak split 2.5, level of detection 0.32, amplitude distance 3, assay variability 0 2.5 3.3, and G values of 2.2, 2.7, 1.9, 1.5, and 1.2.

2.8 | E_2 positive feedback induction of LH surges

RP3V kisspeptin neurons govern the circadian-timed preovulatory LH surge in female rodents[29–31] but the role, if any, of ARC kisspeptin neurons (KNDy neurons) in the LH surge mechanism is less clear.[30] To test whether KNDy neuron depletion affects LH surge secretion in mice, we exposed adult $KNDy^{ABL}$ female mice to a well-established E_2-induced LH surge paradigm. $KNDy^{ABL}$ females sustaining cell ablation in juvenile life and control females were OVX in adulthood (~8 weeks old) and implanted subcutaneously with an E_2 capsule (0.75 μg E_2 in oil) which reliably elicits early evening LH surges 2 days later.[58–60,68] Two days after E_2 implantation, mice in each group were euthanized in either the morning (AM; non-surge time) or the early evening before lights off (PM; time of the LH surge). Blood was collected and left to clot at room temperature for 90 min and then centrifuged to obtain serum. Serum LH levels were assayed by the UVA Ligand Assay Core using a sensitive murine LH sandwich radioimmunoassay (RIA), described below. Using this specific LH sandwich RIA, an LH surge was defined as a PM value >0.70 ng/mL, as in prior surge studies.[59,60]

2.9 | Hormone assays

Tail-tip blood samples were assayed in peri-pubertal mice or adults for LH levels by the University of Virginia Ligand Assay Core using a well-established murine ultra-sensitive (US) LH ELISA[69] which uses a capture monoclonal antibody (anti-bovine LH beta subunit, 518B7; RRID:AB_2665514) and detection polyclonal antibody (rabbit LH antiserum, AFP240580Rb; RRID:AB_2665533). Pre-dilution, the US-LH assay sensitivity is 0.016 ng/mL. As described above, tail-tip blood samples were collected as either 5 μL (PND 28), 4 μL (adults), or 3 μL (LH pulse serial sampling in GDX adults) and then mixed with assay buffer to a final volume of 60 μL; this equates to 12X, 15X, or 20X dilution factors for each assay, respectively. Thus, the functional sensitivities of the US-LH assays were either 0.192 ng/mL (for 5 μL samples), 0.240 ng/mL (for 4 μL samples), or 0.320 ng/mL (for 3 μL samples), with the reportable ranges being 0.192–48.0, 0.240–60.0, and 0.32 to 80.0 ng/mL. All tail-tip and serial sampling LH values are reported as ng/ml of whole blood.

For the assessment of LH surges (E_2 positive feedback), serum LH levels collected in the AM or PM were assayed by the University of Virginia Ligand Assay Core using a well-established, sensitive murine LH sandwich RIA, as in our prior LH studies. This LH sandwich RIA assay uses antibodies against bovine LH and human LH-beta (RRID:AB_2665514, RRID:AB_2665513) and has an assay sensitivity of 0.04 ng/mL with a reportable range: 0.04–75.0 ng/mL.

2.10 | Statistical analysis

Data are reported as mean ± SEM. Data were analyzed and graphed using Prism (GraphPad, San Diego, USA). Mann–Whitney tests were used to compare the 2 groups ($KNDy^{ABL}$ vs. control), with $p < .05$ considered statistically significant. In figures, statistical significance is represented as: *$p < .05$; **$p < .01$, and ***$p < .001$.

3 | RESULTS

3.1 | Confirmation of low RP3V *Kiss1* versus high ARC *Kiss1* cell numbers in juvenile mice

To confirm different developmental numbers of *Kiss1* neurons in the RP3V versus ARC during juvenile life, we used female transgenic $Kiss1^{tdtom}$ mice to count the number of neurons that had ever expressed *Kiss1* at any age up until the juvenile age of PND 12 and compared this with similar analyses at PND 28, a pubertal age. Matching prior reports in rodents measuring active *Kiss1* gene expression at different developmental ages (reviewed in Semaan et al.[6]), we found that on PND 12, the ARC region contained many td-tomato cells, representing *Kiss1* neurons (Figure 1B,C). By contrast, as expected, the RP3V contained virtually no td-tomato cells on PND 12 (Figure 1B,C), with only a very few isolated cells identified in any PND 12 animal, matching similar reports (reviewed in Semaan et al.[6]). Thus, at this early juvenile age, *Kiss1* Cre-driven tdtomato expression has primarily only ever occurred in the ARC kisspeptin population. By the pubertal age of PND 28, ARC tdtomato cells remained abundant but now there were also high numbers of RP3V tdtomato cells (Figure 1C). Comparing PND 28 and PND 12 cell counts indicates that, unlike ARC kisspeptin neurons, virtually all RP3V kisspeptin neurons are born or first start expressing *Kiss1* sometime *after* PND 12, confirming prior findings that active *Kiss1* expression in the RP3V is very low at juvenile ages and starts to increase later in the mid-3rd week of postnatal life.[32,44] Quantitatively, in females, 93% of the total number of PND 28 ARC *Kiss1* cells is already detected at PND 12 whereas <1% of the number of PND 28 RP3V *Kiss1* neurons is detected at PND 12 (Figure 1D, left). Considered another way, only 7% of detectable PND 28 ARC *Kiss1* neurons arise after PND 12 whereas >99% of RP3V *Kiss1* neurons are first detected *after* PND 12 (Figure 1D, right). We strategically capitalized on this major developmental

difference in subsequent experiments to selectively ablate just ARC *Kiss1* neurons before the pubertal period while sparing RP3V *Kiss1* neurons.

3.2 | Confirming targeted depletion of ARC KNDy neurons by DT injection in the juvenile period

To specifically ablate ARC KNDy neurons but not RP3V kisspeptin neurons prior to normal puberty onset, we took advantage of juvenile *Kiss1* gene expression, and hence DTR expression in *Kiss1* Cre+/iDTR$^{fl/fl}$ mice, being restricted to the ARC and not the RP3V. We therefore administered DT to juvenile (PND 12) *Kiss1* Cre+/iDTR$^{fl/fl}$ mice to selectively ablate ARC kisspeptin cells (summarized in Figure 2A). To validate the ablation efficiency of this model, we performed ISH for *Kiss1* mRNA expression in adult brain sections containing the ARC or RP3V from KNDY^{ABL} and control mice. As expected, adult control mice of both sexes had high *Kiss1* expression in the ARC (Figure 2B,C,E,F). By contrast, there was a massive decrease in the number of ARC *Kiss1* (i.e., KNDy) neurons in both male (Figure 2B,C; $p < .001$ vs. control group) and female (Figure 2E,F; $p < .01$ vs. controls) KNDy^{ABL} mice, with only a few scattered residual cells typically present. The mean ablation efficacy of KNDy neurons was approximately 84% and 94% in male and female KNDy^{ABL} mice, respectively (Figure 2D,G), confirming depletion of the majority of KNDy neurons. Unlike the ARC *Kiss1* population, there was no difference in the number of adult RP3V *Kiss1* cells between KNDy^{ABL} and control females (Figure 2H–J), demonstrating that DT injection during the early juvenile period specifically ablated most ARC *Kiss1* neurons but did not alter RP3V *Kiss1* neuron number, as predicted based on the developmental timing of *Kiss1* expression in each region.

3.3 | Juvenile KNDy neuron ablation does not alter BW in either sex

Because metabolic status and BW can significantly alter the timing of puberty onset, we measured BW in male and female KNDy^{ABL} and control mice at the time of DT injection (PND 12), again at weaning (PND 21), and then every 3 days until PND 45. Importantly, for both sexes, there were no group differences in BWs between control and KNDy^{ABL} mice at PND 12, weaning, or any age during the pubertal assessment period (females: Figure 3A–C; males: Figure 3D–F). Thus, the DT treatment did not alter BW in either sex, and KNDy^{ABL} mice demonstrated similar BWs as controls at juvenile, pre-pubertal, peri-pubertal, and young adult ages.

3.4 | Juvenile KNDy neuron ablation delays puberty onset in both sexes

The targeted depletion of ARC kisspeptin neurons in juvenile life allowed us to assess the necessary role of that KNDy cell population in puberty onset. Male and female

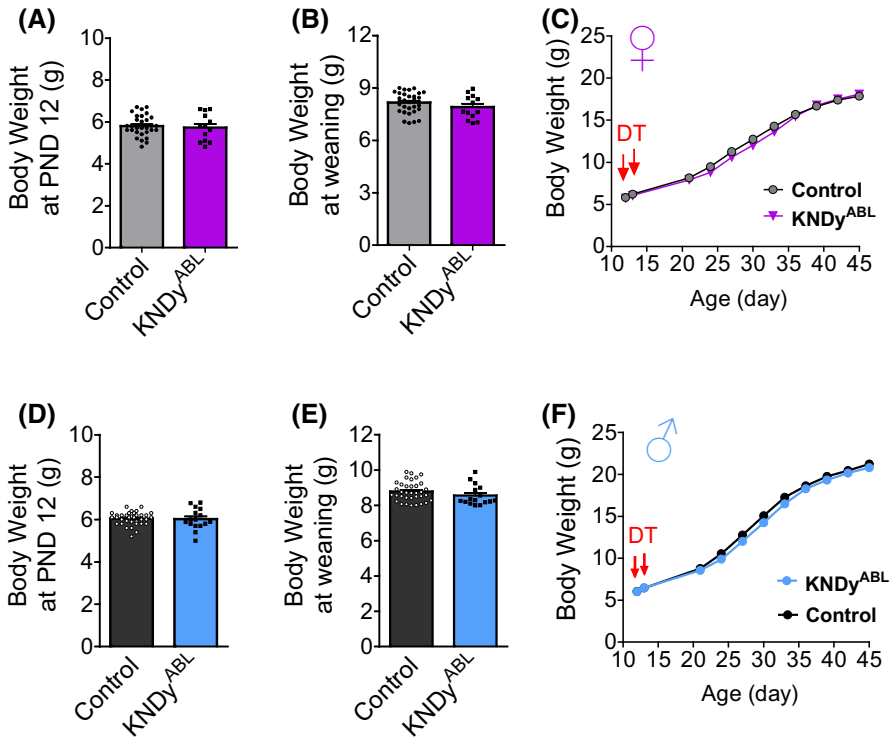

FIGURE 3 BW is normal in mice with ARC *Kiss1* neuron depletion in juvenile life. BWs of female KNDY^{ABL} mice and control littermates at PND 12 (A), at weaning (B), and from PND 12 to 45 (C). Control females: gray bars, circles, and lines; $n = 30$; KNDy^{ABL} females: purple bars, triangles, and lines; $n = 13$. BWs of male KNDY^{ABL} mice and control littermates at PND 12 (D), weaning (E), and from PND 12 to PND 45 (F). Control males: black bars, circles, and lines; $n = 37$; KNDy^{ABL} males: blue bars, circles, and lines; $n = 16$.

KNDy^{ABL} and control mice were checked daily for PS and VO, respectively, beginning at PND 21. Juvenile KNDy neuron ablation resulted in a marked delay in puberty onset in both sexes. Female controls exhibited mean VO around PND 28 (Figure 4A,B), similar to prior studies.[32] By contrast, in KNDy^{ABL} females, there was an obvious and significant delay in VO, by ~5 days on average, compared to control females ($p < .001$, Figure 4A,B). Moreover, the late pubertal measure of FE was also significantly delayed, by ~6 days, in KNDy^{ABL} females vs. controls ($p < .01$; Figure 4D,E). Likewise, in KNDy^{ABL} males, the pubertal measure of PS was significantly delayed, by ~3 days, compared to control males ($p < .001$, Figure 5A,B).

BWs of KNDy^{ABL} mice on the day of VO (Figure 4C) and PS (Figure 5C) were significantly higher ($p < .05$ for each sex) than BWs of control mice on their respective pubertal onset days, reflecting a normal growth curve but later age of puberty onset in KNDy^{ABL} mice. Thus, both male and female mice with depleted numbers of KNDy neurons had significantly delayed pubertal onset compared to controls and this pubertal delay was not due to lower BW or slower growth.

3.5 | Juvenile KNDy neuron ablation reduces peripubertal LH secretion in both sexes

KNDy neurons are thought to govern the downstream secretion of pituitary gonadotropins by direct stimulation of

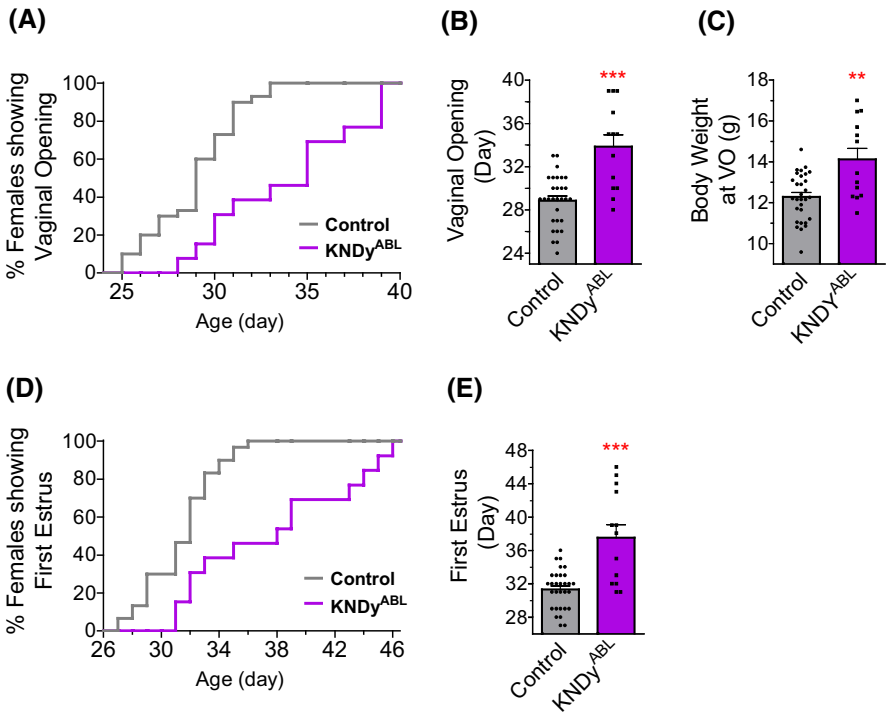

FIGURE 4 ARC *Kiss1* neuron depletion in juvenile life delays puberty onset in females. (A and B) Day of the vaginal opening (VO), a marker of puberty onset in female KNDY^{ABL} mice and control littermates. (C) Mean BWs of female mice on the day of VO. (D and E) Day of first estrus (FE), a later marker of sexual maturation and first ovulation in KNDY^{ABL} and control females. Control females: gray bars, circles, and lines; $n = 30$; KNDy^{ABL} females: purple bars, triangles, and lines; $n = 13$. $^{**}p < .01$; $^{***}p < .001$.

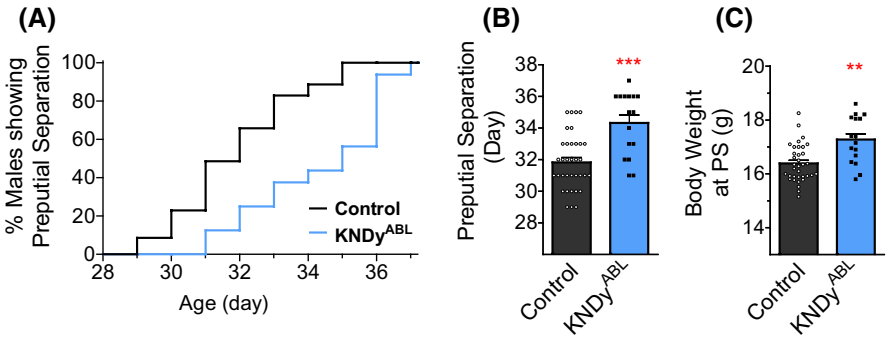

FIGURE 5 ARC *Kiss1* neuron depletion in juvenile life impairs puberty onset in males. (A and B) Day of preputial separation (PS), a marker of puberty onset in KNDY^{ABL} and control males. (C) BWs of male mice on the day of PS. Control males: black bars, circles, and lines; $n = 37$; KNDy^{ABL} males: blue bars, circles, and lines; $n = 16$. $^{**}p < .01$; $^{***}p < .001$.

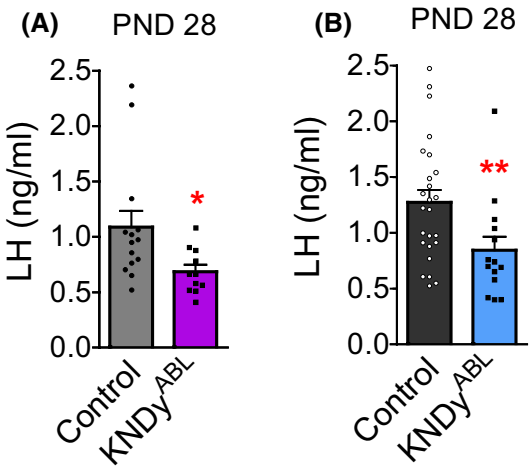

FIGURE 6 ARC *Kiss1* neuron depletion in juvenile life decreases peripubertal LH secretion in both sexes. Basal LH levels in (A) female mice during the peripubertal period on PND 28 ($n = 11–14$/group) and (B) peripubertal males at PND 28 ($n = 14–25$/group). $^{**}p < .01$; $^{*}p < .05$.

GnRH secretion.[9] Thus, KNDy neuron depletion would be expected to reduce downstream GnRH and LH secretion, which could underlie the observed delays in pubertal onset in both sexes. To ascertain whether juvenile ablation of most KNDy neurons impacted pubertal LH secretion, we measured peri-pubertal LH levels in the blood as a one-off measure on PND 28. Blood LH levels at PND 28 were significantly lower in KNDy^{ABL} females compared to control females ($p < .05$; Figure 6A). Like females, peripubertal KNDy^{ABL} male mice also had significantly lower LH levels at PND 28 versus similarly-aged control males ($p < .01$; Figure 6B). Overall, these lower LH levels during the peripubertal period match the observed delay in pubertal onset in KNDy^{ABL} mice of each sex.

3.6 | Juvenile KNDy neuron ablation reduces basal LH secretion in adulthood but not the downstream LH response to kisspeptin signaling

Given the lower LH levels detected in peripubertal KNDy^{ABL} mice, we also assessed basal LH levels in KNDy^{ABL} mice later in adulthood. Similar to findings on PND 28, adult females (diestrus stage) that had sustained KNDy^{ABL} earlier in juvenile life had significantly lower basal LH levels than adult diestrus control females ($p < .05$; Figure 7A). To confirm that the observed reductions in LH levels in KNDy^{ABL} females were not due to impairment at the level of their GnRH neurons or pituitary cells, we challenged adult diestrus females with kisspeptin to interrogate their functional LH secretory response. The evoked concentration of LH secreted in KNDy^{ABL} females

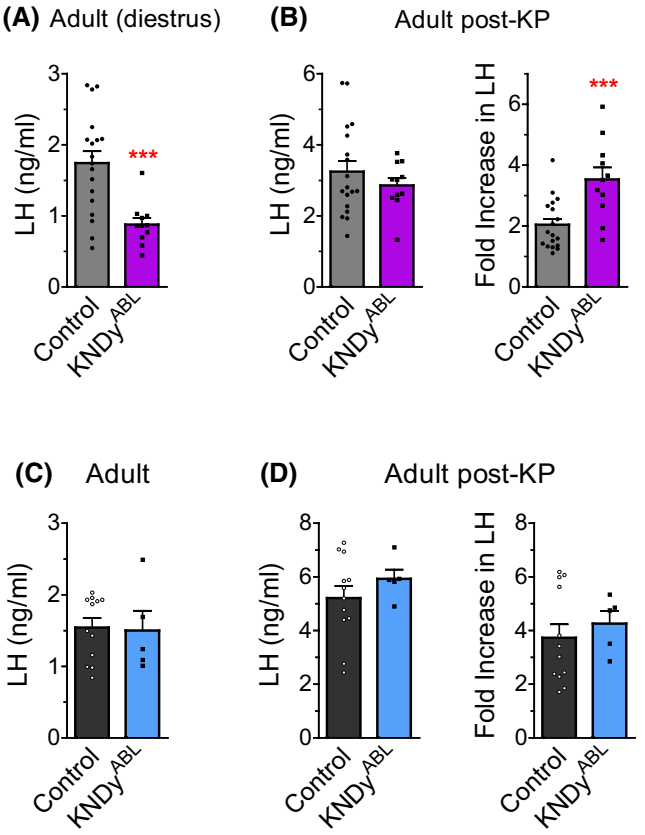

FIGURE 7 ARC *Kiss1* neuron depletion in juvenile life decreases adult female basal LH levels but not GnRH or gonadotrope functionality. (A) Basal LH levels in adult diestrus female mice. (B) LH levels (*left*) and fold-increase in LH (*right*) in diestrus females after exogenous kisspeptin (Kp-10) administration to test the functionality of downstream GnRH neurons ($n = 10–18$/group). (C) Basal LH levels in adult male mice. (D) LH levels (*left*) and fold-increase in LH (*right*) in adult males after exogenous kisspeptin (Kp-10) injection ($n = 5–12$/group). $^{***}p < .001$ versus controls.

in response to the in vivo kisspeptin challenge was not different from that of control females (Figure 7B left), indicating that downstream GnRH neuron and pituitary function is still functional in KNDy^{ABL} females, as predicted. The fold-increase in LH induced by kisspeptin injection from basal levels was significantly higher in KNDy^{ABL} females than controls (Figure 7B right), likely reflecting a significantly lower basal starting level in the former group (see Figure 7A).

As with females, we also measured basal LH levels in adult males who had sustained KNDy^{ABL} earlier in juvenile life. Surprisingly, despite our previous observation of significantly lower LH in KNDy^{ABL} males on PND 28, basal LH was not different between adult KNDy^{ABL} and control males (Figure 7C), unlike adult KNDy^{ABL} females who showed reduced basal LH levels. As with females, the functional LH secretory response after the kisspeptin challenge, testing downstream GnRH and

gonadotrope function, was similar between adult male groups (Figure 7D).

3.7 | Juvenile KNDy neuron depletion impairs adult LH pulse concentrations but not LH pulse frequency

The surprising lack of difference in basal LH levels in adult KNDy^{ABL} and control males may possibly reflect a technical limitation of the one-time random blood sampling at an unknown phase of each male's slow LH pulse pattern. To resolve this issue, we conducted a serial sampling experiment to determine whether juvenile depletion of KNDy neurons reduces parameters of adult pulsatile LH secretion in KNDy^{ABL} males. KNDy^{ABL} and control males were GDX in adulthood, several weeks before blood sampling, to remove negative feedback and amplify the frequency and concentration of endogenous LH pulses. As expected, control males exhibited rapid, high-amplitude LH pulses with high basal LH levels and high pulse peak concentrations (Figure 8A). By contrast, KNDy^{ABL} males demonstrated a pattern of significantly diminished LH secretion versus control males (Figure 8A). Overall mean LH levels during the serial sampling period were significantly lower, by 33%, in KNDy^{ABL} versus control males ($p < .001$, Figure 8B). Analysis of specific pulse secretion parameters indicated no change in LH pulse frequency (Figure 8C) but a significant decrease in both basal LH levels (by 37%) and peak LH levels (by 29%) in KNDy^{ABL} males versus control males ($p < .001$ for each measure vs. controls; Figure 8D,E). Because basal and pulse peak levels were both reduced by a similar magnitude, the mean pulse amplitude was not different between groups (Figure 8F). Thus, juvenile depletion of a majority of KNDy neurons significantly lowers overall LH pulse secretion concentrations in adulthood, but does not change pulse frequency or pulse amplitude.

3.8 | Selective KNDy neuron depletion does not alter LH surges in mice

In female rodents, a large E_2-induced LH surge on the evening of proestrus triggers ovulation. RP3V kisspeptin neurons participate in the generation of LH surges,[30] whereas the involvement of KNDy neurons in the LH surge event remains unclear. Ablation studies in adult female rats report some influence of KNDy neurons on the LH surge magnitude,[51–53] suggesting that our KNDy^{ABL} mice might have altered LH surges. We therefore tested whether a complete KNDy neuron population is necessary for proper E_2 induction of the LH surge in KNDy^{ABL} females. As expected, control OVX + E_2 females had very low LH in the AM, when the surge does not normally occur, and produced high LH levels in the PM, reflecting typical LH surges (Figure 9). In OVX + E_2 KNDy^{ABL} females, LH levels were similar to the controls during both the AM and PM times (Figure 9A). Indeed, KNDy^{ABL} females demonstrated very low LH levels in the AM and high LH surge levels in the PM ($p < .001$ vs. their AM values), with no difference in surge magnitude versus control females (Figure 9A). The percentage of females generating a detectable LH surge was also not statistically different between KNDy^{ABL} and control females (Figure 9B). These results demonstrate that ablation of most KNDy neurons does not affect the occurrence or magnitude of the LH surge in mice and is consistent with the observed normal numbers of RP3V *Kiss1* neurons in KNDy^{ABL} females (Figure 2G–I).

4 | DISCUSSION

The neural mechanisms timing the onset of pubertal activation of the reproductive axis are still not completely understood, nor are the specific cellular populations in the brain that govern this process. This study interrogated the functional contribution of just the ARC kisspeptin neural population to the pubertal process. Kisspeptin is implicated in triggering puberty onset but the specific population of kisspeptin neurons responsible for driving pubertal timing has not previously been determined. We addressed this with targeted DT-mediated ablation of just ARC kisspeptin neurons (KNDy neurons) in mice at a juvenile age when only ARC *Kiss1* neurons are abundantly present but >99% of RP3V *Kiss1* neurons are still absent.[32,44,70–72] Because this selective ablation occurred during the juvenile period, it permitted subsequent study of puberty onset which was not previously possible with prior studies ablating KNDy cells only in adulthood.[50–53] Our approach also avoided developmental compensation and neural circuit reorganization that might occur during in-utero or perinatal development in some transgenic knockout lines.[49,73] Our findings demonstrate that selective, partial depletion of most ARC kisspeptin cells in early juvenile development results in significant delays in puberty onset in both sexes, implicating an intact KNDy neuron cell population as being necessary for normal pubertal timing in mice.

Although kisspeptin and NKB signaling are widely-believed to govern puberty onset,[3,54] the specific kisspeptin and NKB cell population(s) necessary for normal pubertal activation have not been definitively established. We previously hypothesized that observed peri-pubertal increases in *Kiss1* and *Tac2* expression in the ARC[6,33]

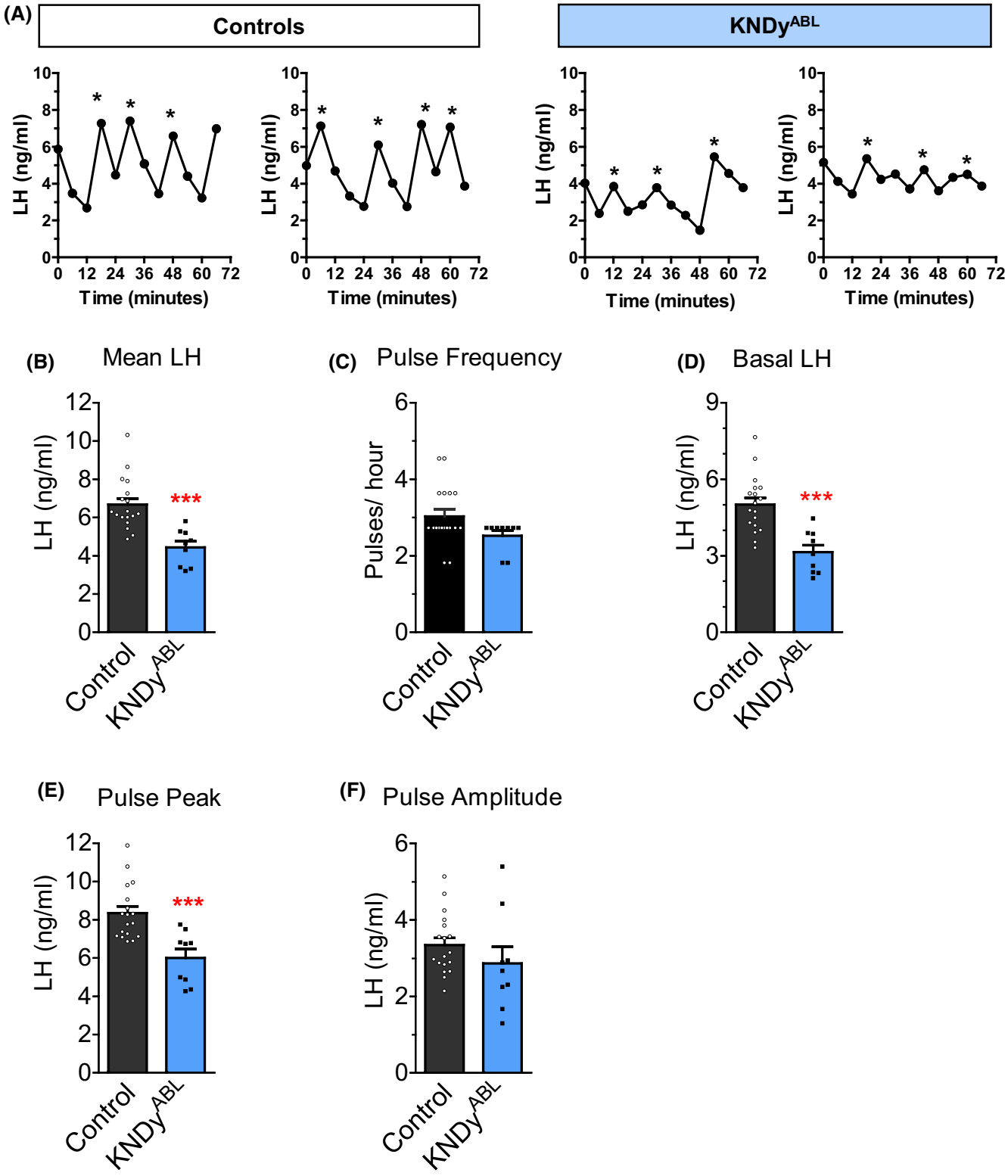

FIGURE 8 Selective ARC *Kiss1* neuron depletion decreases pulsatile LH secretion levels. (A) Representative examples of in vivo LH pulse secretion in awake, adult GDX control and KNDy^{ABL} males. (B) Overall mean LH during the serial sampling was significantly lower in KNDy^{ABL} males than in control males ($n = 9–18$/group). (C–F) Analysis of individual LH pulse secretion parameters revealed significant decreases in KNDy^{ABL} mice in basal LH levels (D) and pulse peak LH values (E), with no change in pulse frequency (C) or pulse amplitude (F). *$p < .05$, ***$p < .001$.

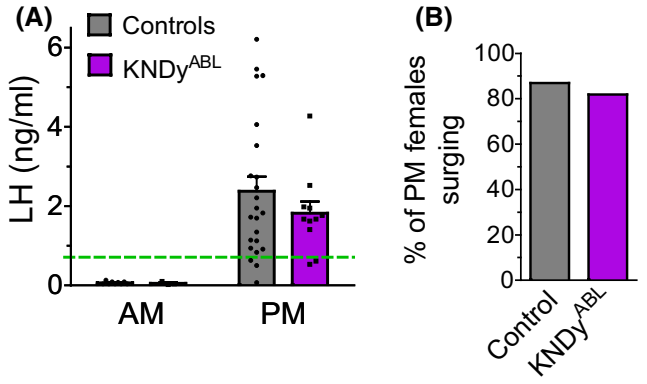

FIGURE 9 ARC *Kiss1* neuron depletion does not alter generation or magnitude of the LH surge. (A) Serum LH levels in adult OVX + E_2 females euthanized in the morning (AM, time when the LH surge does not occur; $n = 3–9$/group) or in the evening just before lights off (PM; circadian time of the LH surge; $n = 11–23$/group). In response to E_2 treatment, both $KNDY^{ABL}$ and control females show robust elevations in PM LH levels, indicative of the LH surge. Green dotted line designates the LH surge threshold when using the mouse LH sandwich RIA assay (0.70 ng/mL). (B) The percentage of females in each group successfully displaying an E_2-induced LH surge did not differ between groups.

reflect the KNDy cell population achieving stronger capability to drive downstream GnRH and LH pulses, thereby helping to initiate pubertal activation of the reproductive axis. This notion is supported by delayed puberty onset in female rodents with experimentally reduced *Kiss1* or *Tac2* mRNA levels in the ARC.[74–77] However, while those prior studies specifically altered just *Kiss1* or *Tac2* gene expression, the potential pubertal role of the KNDy cell population itself, as a whole, was not previously studied. We tested this here by selectively depleting most KNDy neurons while sparing RP3V kisspeptin neurons to see if 1) KNDy neurons are necessary for normal pubertal timing, and indirectly 2) if RP3V kisspeptin neurons are sufficient, in the absence of most ARC kisspeptin cells, to drive normal pubertal timing. We found that selective depletion of the vast majority of KNDy neurons strongly delayed puberty onset and lowered peripubertal LH in both sexes. The observed pubertal delay was somewhat larger in females than males, perhaps reflecting the slightly greater efficiency of cell ablation in females (94% vs. 84%). In both sexes, puberty was not completely prevented, as all mice eventually displayed pubertal onset. This is likely due to a combination of some small retained stimulatory input over time from the few (~6–16%) residual, intact KNDy neurons along with possible involvement of non-KNDy modulation of GnRH neurons, as was suggested can occur in several prior knockout studies.[15,26,78,79] Regardless, our findings indicate that ARC kisspeptin neurons play an important and necessary role in the normal increase in peri-pubertal LH secretion and properly-timed initiation of puberty in both sexes.

The delay in pubertal onset in $KNDy^{ABL}$ mice was not linked to a reduction in BW or metabolic disturbance, as our design only included pups from within a narrow litter size and BW range to avoid known confounding effects of different litter sizes and BWs on pubertal tempo.[62,80,81] Indeed, BWs were not different between groups at juvenile, pubertal, or young adult ages, indicating that the observed pubertal delays in $KNDy^{ABL}$ mice were not due to lower BWs.

Our present findings did not experimentally alter RP3V kisspeptin neurons, and we cannot rule out that similar pre-pubertal ablation of just the RP3V kisspeptin population might also alter pubertal timing in one or both sexes. Indeed, the role of each kisspeptin population in the control of puberty is not mutually exclusive. However, contrasting ARC kisspeptin neurons' known role in stimulating GnRH and LH pulses, the primary function of RP3V kisspeptin neurons is thought to be the generation of E2-induced LH surges in adult females, and it is unclear if RP3V kisspeptin neurons also control pubertal onset. This is especially true for males, given the highly sexually dimorphic nature of this population such that only a few RP3V kisspeptin cells are present in either pubertal or adult males.[82] Based on these considerations, along with the known ability of E_2 to increase *Kiss1* expression in the RP3V, it is possible that the previously observed pubertal increase in RP3V *Kiss1* levels may be a consequence of rising gonadal sex steroids caused by pubertal activation, rather than a independent event that itself triggers puberty onset.[6,33] Regardless, while ARC kisspeptin neurons may govern the timing of pubertal *onset*, RP3V kisspeptin neurons may have a critical role in the *later* stage of pubertal progression in females, specifically for the timing of FE, a late-pubertal marker reflecting the first ovulation.[7] Future studies either selectively ablating a majority of RP3V kisspeptin neurons before puberty onset or selectively silencing RP3V kisspeptin neuron activity during puberty are needed to assess this possibility. Intriguingly, a prior study in female rats selectively knocked down *Kiss1* expression in the RP3V and reported a delay in VO,[83] despite only a moderate gene knockdown magnitude of 36% and unaffected ARC *Kiss1* levels. This interesting outcome suggests that kisspeptin signaling arising from the RP3V may somehow be involved in pubertal timing, at least in female rats. Exactly how RP3V kisspeptin release might alter pubertal GnRH pulse secretion (which initiates puberty onset) is currently unknown and whether this also occurs in males, which lack a robust RP3V kisspeptin population, or other species is an important future area of study.

Our finding of delayed puberty after juvenile KNDy neuron depletion differs from a study in which transgenic mice with selective *Kiss1* gene knockout in dynorphin neurons (which co-express *Kiss1* in ARC KNDy neurons) surprisingly had normal puberty onset.[84] While caution is warranted when comparing the different technical approaches and interpretations of *cell ablation* versus *gene knockdown*, one likely explanation for the normal puberty in the $Pdyn^{CRE}/Kiss^{FLOX}$ mice is that the timing of *Kiss1* deletion in that model is dependent on sufficient ARC *Pdyn* expression to drive Cre recombinase expression. While *Pdyn* expression in the ARC, and specifically in KNDy neurons, is high in adult animals, such *Pdyn* levels are much lower before puberty. Indeed, in pilot ISH assays run during several prior pubertal projects,[32,85] we observed only very low *Pdyn* levels in the ARC of juvenile and prepubertal mice (A.S. Kauffman, unpublished observations), and other labs have similarly reported that ARC *Pdyn* or DYN-ir levels are low before puberty and rise markedly during or after puberty.[86–91] Thus, deletion of the ARC *Kiss1* gene may not have occurred yet or might have been incomplete by the pubertal period in $Pdyn^{CRE}/Kiss^{FLOX}$ mice, precluding a significant change in pubertal timing in that study. The degree of ARC *Kiss1* knockout was only quantified in adult $Pdyn^{CRE}/Kiss^{FLOX}$ mice (~85–95%),[84] so it remains unknown whether their normal puberty timing was due to insufficient *Kiss1* knockout at earlier peri-pubertal ages. To this point, another study from Nagae et al.,[92] knocked down *Kiss1* gene expression in both the ARC and RP3V by crossing $Oprk1^{CRE}$ rats (*Oprk1* encodes kappa opioid receptor) with $Kiss^{FLOX}$ rats: $Oprk1^{CRE+}/Kiss^{FLOX}$ rats showed disrupted LH pulse and surge secretion in adulthood, but pubertal onset was unaffected. Those authors noted that the normal puberty correlated with much lower *Oprk* + *Kiss1* co-expression in pubertal versus adult rats, indicating that most *Kiss1* deletion in $Oprk1^{CRE+}/Kiss^{FLOX}$ rats occurred after puberty.[92]

The fact that puberty was delayed in $KNDy^{ABL}$ mice of both sexes signifies that KNDy ablation occurred at some point before the normal pubertal period. While the present study did not determine the specific time duration for KNDy neurons to be ablated following DT treatment on PND 12, multiple prior studies in other iDTR mouse models indicate cell death is induced within 24–48 h of DT injection and physiological or behavioral effects observed as soon as 48–72 h following DT.[93–101] We therefore predict that KNDy ablation most likely occurred by 48–72 h post-DT treatment on PND 12 (i.e., by PND 15), well before normal pubertal onset typically occurs (~PND 28–30), though the actual timing was not studied.

KNDy neurons govern downstream GnRH and LH pulse secretion,[9,56] but surprisingly LH pulse secretion was not studied in prior KNDy ablation studies.[50–53] In the present study, depletion of most, but not all, KNDy neurons induced significant decreases in LH secretion levels, both for one-off measures in peri-pubertal mice and for basal and peak LH parameters of LH pulse secretion in adults. These findings support our prediction that the presence of fewer KNDy neurons in $KNDy^{ABL}$ mice would result in less overall kisspeptin released to stimulate GnRH and LH secretion. Interestingly, LH pulse frequency was not altered in $KNDy^{ABL}$ mice, suggesting that the small remaining network of few non-ablated KNDy cells was sufficient to functionally maintain the regular pulse generator timing without altered frequency. Thus, while the timing of LH pulses was unchanged, overall levels of LH were reduced; the few KNDy neurons remaining therefore appear sufficient for some low degree of GnRH stimulation that results in low, but not absent, LH levels. We also confirmed that LH secretion in $KNDy^{ABL}$ mice of both sexes was normal following exogenous kisspeptin treatment, indicating that GnRH neurons and downstream gonadotropes retain fully functional capabilities when most KNDy cells are absent. Although no prior KNDy ablation study measured in vivo LH pulse patterns, one such study sampled LH every 30 min in adult GDX female rats and reported no change in mean LH levels after KNDy ablation.[52] However, the degree of KNDy ablation in that study was ~66% whereas it was 84% in our $KNDy^{ABL}$ males and 94% in $KNDy^{ABL}$ females; the larger degrees of cell ablation in the present study may explain our observed reductions in LH concentrations. Relatedly, Nagae et al.[102] used AAV technology to show that recovery of at least 20% of the KNDy neuron population is sufficient to display LH pulses in adult *Kiss1* KO female rats, suggesting that a threshold of ≥20% of KNDy neurons may be required for a functional reproductive axis. Although it is unknown if the same threshold level exists for mice (or males), our $KNDy^{ABL}$ mice had less than 20% of KNDy neurons remaining (on average, just 6% in females and 16% in males) and displayed reduced LH levels and delayed puberty, supporting Nagae et al.'s conclusions.[102] Relatedly, in a different study, LH pulses were still present in female rats in which the majority of *Kiss1* in the ARC and half the *Kiss1* in the RP3V was knocked out, suggesting that the remaining small portion of ARC *Kiss1*-expressing neurons are sufficient to maintain some degree of LH pulse generation.[92]

Along with pulsatile LH secretion throughout most of their cycle, females also exhibit an E_2-mediated LH surge on proestrus ("positive feedback"), thereby triggering ovulation. RP3V kisspeptin neurons are key players in the generation of LH surges,[29–31] supported in part by findings of increased RP3V *Kiss1* mRNA and neuron activation during the surge[59,103,104] and sufficiency of progesterone receptors in RP3V kisspeptin neurons for surge

occurrence.[58,60] In the present study, KNDy neuron ablation initiated in early juvenile life did not affect adult RP3V *Kiss1* cell numbers, suggesting that the ability to generate LH surges should be unaffected. However, limited prior evidence suggested the possible involvement of KNDy neurons themselves in the LH surge process: some KNDy neurons project to the RP3V region[11,105,106] and adult female rats sustaining partial ablation of KNDy neurons displayed increased LH surge levels,[51–53] suggesting that KNDy neurons may normally act to *reduce* LH surge magnitude. Conversely, an optogenetic study in mice suggested that activation of KNDy neurons is sufficient to *stimulate* RP3V kisspeptin neurons[11] and possibly *amplify* the magnitude of the LH surge,[107] rather than reduce it as in rats, leaving this issue unresolved. Here, we found that adult female KNDy^{ABL} mice with most of their KNDy neurons depleted displayed no alteration in either the occurrence of LH surges or the LH surge magnitude, suggesting that the majority of the KNDy neuron population is not required for normal E_2-triggered LH surges in mice. Whether the few remaining non-ablated KNDy neurons are sufficient on their own to provide full modulation of the LH surge magnitude cannot be ruled out and whether the different LH surge outcomes between KNDy ablated rats and our present KNDy ablated mice reflect species and/or methodological differences is unknown. Regardless, our data suggest that the majority of KNDy neurons are not required for a normal LH surge response to E_2 positive feedback in mice.

In conclusion, our findings establish a requisite role of the KNDy neuron population in the timing of normal puberty onset in both sexes. This study selectively depleted the majority of KNDy neurons *before* the peri-pubertal period, enabling examination of subsequent pubertal measures without a caveat of developmental compensation on neural circuits. Selective juvenile ablation of most KNDy neurons significantly delayed puberty onset and lowered peri-pubertal LH in both sexes, demonstrating that a complete KNDy neuron population is necessary for normally-timed puberty onset. We also determined that juvenile KNDy neuron ablation impaired the levels of LH pulse secretion studied later in adult castrated mice, reducing basal and peak LH concentrations without altering the pulse frequency timing mechanism. These findings support the current model of KNDy neuron participation in governing LH pulse secretion[27,55] and suggest that only a small subset of KNDy neurons is needed for dictating normal LH pulse frequency. Finally, we show that, unlike in female rats, a complete KNDy neuron population in female mice is not essential for normal E_2-induced LH surge occurrence or normal magnitude of LH surges.

AUTHOR CONTRIBUTIONS

E. Coutinho and A. Kauffman conceived and designed the research; E. Coutinho, L. Esparza, P. Steffen, R. Liaw, and S. Bolleddu performed the research and acquired the data. E. Coutinho, L. Esparza, and A. Kauffman analyzed the data. E. Coutinho and A. Kauffman interpreted the data and made figures. E. Coutinho and A. Kauffman wrote and revised the manuscript.

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DISCLOSURES

The authors declare no competing financial interests.

DATA AVAILABILITY STATEMENT

The data presented in the findings of this study will be available from the corresponding author upon reasonable request and deposited in the DRYAD repository (doi: 10.5061/dryad.rn8pk0pmz).

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REFERENCES

1. Herbison AE. Control of puberty onset and fertility by gonadotropin-releasing hormone neurons. *Nat Rev Endocrinol*. 2016;12(8):452-466.
2. Terasawa E, Guerriero KA, Plant TM. Kisspeptin and puberty in mammals. *Adv Exp Med Biol*. 2013;784:253-273.
3. Ojeda SR, Skinner MK. Puberty in the rat. In: Neill JD, ed. *Knobil and Niell's Physiology of Reproduction*. 3rd ed. Elsevier; 2006:2061-2126.
4. Harris GC, Levine JE. Pubertal acceleration of pulsatile gonadotropin-releasing hormone release in male rats as revealed by microdialysis. *Endocrinology*. 2003;144(1):163-171.
5. Sisk CL, Richardson HN, Chappell PE, Levine JE. In vivo gonadotropin-releasing hormone secretion in female rats during peripubertal development and on proestrus. *Endocrinology*. 2001;142(7):2929-2936.
6. Semaan SJ, Tolson KP, Kauffman AS. The development of kisspeptin circuits in the mammalian brain. *Adv Exp Med Biol*. 2013;784:221-252.
7. Mayer C, Acosta-Martinez M, Dubois SL, et al. Timing and completion of puberty in female mice depend on estrogen

receptor alpha-signaling in kisspeptin neurons. *Proc Natl Acad Sci USA*. 2010;107(52):22693-22698.
8. Han SK, Gottsch ML, Lee KJ, et al. Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty. *J Neurosci*. 2005;25(49):11349-11356.
9. Clarkson J, Han SY, Piet R, et al. Definition of the hypothalamic GnRH pulse generator in mice. *Proc Natl Acad Sci USA*. 2017;114(47):E10216-e10223.
10. Piet R, Kalil B, McLennan T, Porteous R, Czieselsky K, Herbison AE. Dominant neuropeptide Cotransmission in Kisspeptin-GABA regulation of GnRH neuron firing driving ovulation. *J Neurosci*. 2018;38(28):6310-6322.
11. Qiu J, Nestor CC, Zhang C, et al. High-frequency stimulation-induced peptide release synchronizes arcuate kisspeptin neurons and excites GnRH neurons. *elife*. 2016;5:e16246.
12. Topaloglu AK, Tello JA, Kotan LD, et al. Inactivating KISS1 mutation and hypogonadotropic hypogonadism. *N Engl J Med*. 2012;366(7):629-635.
13. de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc Natl Acad Sci USA*. 2003;100(19):10972-10976.
14. Seminara SB, Messager S, Chatzidaki EE, et al. The GPR54 gene as a regulator of puberty. *N Engl J Med*. 2003;349(17):1614-1627.
15. Lapatto R, Pallais JC, Zhang D, et al. Kiss1−/− mice exhibit more variable hypogonadism than Gpr54−/− mice. *Endocrinology*. 2007;148(10):4927-4936.
16. Uenoyama Y, Nakamura S, Hayakawa Y, et al. Lack of pulse and surge modes and glutamatergic stimulation of luteinising hormone release in Kiss1 knockout rats. *J Neuroendocrinol*. 2015;27(3):187-197.
17. Pineda R, Garcia-Galiano D, Roseweir A, et al. Critical roles of kisspeptins in female puberty and preovulatory gonadotropin surges as revealed by a novel antagonist. *Endocrinology*. 2010;151(2):722-730.
18. Goodman RL, Lehman MN, Smith JT, et al. Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin a and neurokinin B. *Endocrinology*. 2007;148(12):5752-5760.
19. Wakabayashi Y, Nakada T, Murata K, et al. Neurokinin B and dynorphin a in kisspeptin neurons of the arcuate nucleus participate in generation of periodic oscillation of neural activity driving pulsatile gonadotropin-releasing hormone secretion in the goat. *J Neurosci*. 2010;30(8):3124-3132.
20. Cheng G, Coolen LM, Padmanabhan V, Goodman RL, Lehman MN. The kisspeptin/neurokinin B/dynorphin (KNDy) cell population of the arcuate nucleus: sex differences and effects of prenatal testosterone in the sheep. *Endocrinology*. 2010;151:301-311.
21. Young J, Bouligand J, Francou B, et al. TAC3 and TACR3 defects cause hypothalamic congenital hypogonadotropic hypogonadism in humans. *J Clin Endocrinol Metab*. 2010;95(5):2287-2295.
22. Topaloglu AK, Reimann F, Guclu M, et al. TAC3 and TACR3 mutations in familial hypogonadotropic hypogonadism reveal a key role for neurokinin B in the central control of reproduction. *Nat Genet*. 2009;41(3):354-358.
23. Ramaswamy S, Seminara SB, Plant TM. Evidence from the agonadal juvenile male rhesus monkey (Macaca mulatta) for the view that the action of neurokinin B to trigger gonadotropin-releasing hormone release is upstream from the kisspeptin receptor. *Neuroendocrinology*. 2011;94(3):237-245.
24. Ramaswamy S, Seminara SB, Ali B, Ciofi P, Amin NA, Plant TM. Neurokinin B stimulates GnRH release in the male monkey (Macaca mulatta) and is colocalized with kisspeptin in the arcuate nucleus. *Endocrinology*. 2010;151(9):4494-4503.
25. Garcia JP, Guerriero KA, Keen KL, Kenealy BP, Seminara SB, Terasawa E. Kisspeptin and neurokinin B signaling network underlies the pubertal increase in GnRH release in female rhesus monkeys. *Endocrinology*. 2017;158(10):3269-3280.
26. True C, Nasrin Alam S, Cox K, Chan YM, Seminara SB. Neurokinin B is critical for normal timing of sexual maturation but dispensable for adult reproductive function in female mice. *Endocrinology*. 2015;156(4):1386-1397.
27. Herbison AE. The gonadotropin-releasing hormone pulse generator. *Endocrinology*. 2018;159(11):3723-3736.
28. Han SY, Morris PG, Kim JC, et al. Mechanism of kisspeptin neuron synchronization for pulsatile hormone secretion in male mice. *Cell Rep*. 2023;42(1):111914.
29. Herbison AE. Estrogen positive feedback to gonadotropin-releasing hormone (GnRH) neurons in the rodent: the case for the rostral periventricular area of the third ventricle (RP3V). *Brain Res Rev*. 2008;57(2):277-287.
30. Kauffman AS. Neuroendocrine mechanisms underlying estrogen positive feedback and the LH surge. *Front Neurosci*. 2022;16:953252.
31. Piet R. Circadian and kisspeptin regulation of the preovulatory surge. *Peptides*. 2023;163:170981.
32. Semaan SJ, Kauffman AS. Daily successive changes in reproductive gene expression and neuronal activation in the brains of pubertal female mice. *Mol Cell Endocrinol*. 2015;401:84-97.
33. Semaan SJ, Kauffman AS. 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. *Mol Cell Endocrinol*. 2022;551:111654.
34. Alfaia C, Robert V, Poissenot K, et al. Sexually dimorphic gene expression and neurite sensitivity to estradiol in fetal arcuate Kiss1 cells. *J Endocrinol*. 2020;244(2):273-283.
35. Desroziers E, Droguerre M, Bentsen AH, et al. Embryonic development of kisspeptin neurones in rat. *J Neuroendocrinol*. 2012;24(10):1284-1295.
36. Kumar D, Periasamy V, Freese M, Voigt A, Boehm U. In utero development of Kisspeptin/GnRH neural circuitry in male mice. *Endocrinology*. 2015;156(9):3084-3090.
37. Kumar D, Freese M, Drexler D, Hermans-Borgmeyer I, Marquardt A, Boehm U. Murine arcuate nucleus kisspeptin neurons communicate with GnRH neurons in utero. *J Neurosci*. 2014;34(10):3756-3766.
38. Takumi K, Iijima N, Ozawa H. Developmental changes in the expression of kisspeptin mRNA in rat hypothalamus. *J Mol Neurosci*. 2011;43(2):138-145.
39. Cao J, Patisaul HB. Sexually dimorphic expression of hypothalamic estrogen receptors alpha and beta and Kiss1 in neonatal male and female rats. *J Comp Neurol*. 2011;519(15):2954-2977.
40. Cao J, Mickens JA, McCaffrey KA, Leyrer SM, Patisaul HB. Neonatal bisphenol a exposure alters sexually dimorphic gene expression in the postnatal rat hypothalamus. *Neurotoxicology*. 2012;33(1):23-36.

41. Takase K, Uenoyama Y, Inoue N, et al. Possible role of oestrogen in pubertal increase of Kiss1/kisspeptin expression in discrete hypothalamic areas of female rats. *J Neuroendocrinol*. 2009;21(6):527-537.
42. Gill JC, Navarro VM, Kwong C, et al. Increased neurokinin B (Tac2) expression in the mouse arcuate nucleus is an early marker of pubertal onset with differential sensitivity to sex steroid-negative feedback than Kiss1. *Endocrinology*. 2012;153(10):4883-4893.
43. Desroziers E, Mikkelsen JD, Duittoz A, Franceschini I. Kisspeptin-immunoreactivity changes in a sex- and hypothalamic-region-specific manner across rat postnatal development. *J Neuroendocrinol*. 2012;24(8):1154-1165.
44. Semaan SJ, Murray EK, Poling MC, Dhamija S, Forger NG, Kauffman AS. BAX-dependent and BAX-independent regulation of Kiss1 neuron development in mice. *Endocrinology*. 2010;151:5807-5817.
45. Clarkson J, Herbison AE. Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons. *Endocrinology*. 2006;147(12):5817-5825.
46. Clarkson J, Boon WC, Simpson ER, Herbison AE. Postnatal development of an estradiol-kisspeptin positive feedback mechanism implicated in puberty onset. *Endocrinology*. 2009;150(7):3214-3220.
47. Gill JC, Wang O, Kakar S, Martinelli E, Carroll RS, Kaiser UB. Reproductive hormone-dependent and -independent contributions to developmental changes in kisspeptin in GnRH-deficient hypogonadal mice. *PLoS One*. 2010;5(7):e11911.
48. Naule L, Robert V, Parmentier C, et al. Delayed pubertal onset and prepubertal Kiss1 expression in female mice lacking central oestrogen receptor beta. *Hum Mol Genet*. 2015;24(25):7326-7338.
49. Mayer C, Boehm U. Female reproductive maturation in the absence of kisspeptin/GPR54 signaling. *Nat Neurosci*. 2011;14(6):704-710.
50. Mittelman-Smith MA, Williams H, Krajewski-Hall SJ, et al. Arcuate kisspeptin/neurokinin B/dynorphin (KNDy) neurons mediate the estrogen suppression of gonadotropin secretion and body weight. *Endocrinology*. 2012;153(6):2800-2812.
51. Helena CV, Toporikova N, Kalil B, et al. KNDy neurons modulate the magnitude of the steroid-induced luteinizing hormone surges in Ovariectomized rats. *Endocrinology*. 2015;156(11):4200-4213.
52. Campideli-Santana AC, Gusmao DO, Almeida F, Araujo-Lopes R, Szawka RE. Partial loss of arcuate kisspeptin neurons in female rats stimulates luteinizing hormone and decreases prolactin secretion induced by estradiol. *J Neuroendocrinol*. 2022;34(11):e13204.
53. Mittelman-Smith MA, Krajewski-Hall SJ, McMullen NT, Rance NE. Ablation of KNDy neurons results in hypogonadotropic hypogonadism and amplifies the steroid-induced LH surge in female rats. *Endocrinology*. 2016;157(5):2015-2027.
54. Plant TM, Witchel SM. Puberty in non-human primates and humans. In: Neill JD, ed. *Knobil and Neill's Physiology of Reproduction*. 3rd ed. Elsevier; 2006:2177-2230.
55. Goodman RL, Herbison AE, Lehman MN, Navarro VM. Neuroendocrine control of gonadotropin-releasing hormone: pulsatile and surge modes of secretion. *J Neuroendocrinol*. 2022;34:e13094, Neuroendocrine control of gonadotropin-releasing hormone: Pulsatile and surge modes of secretion.
56. Han SY, Kane G, Cheong I, Herbison AE. Characterization of GnRH pulse generator activity in male mice using GCaMP fiber photometry. *Endocrinology*. 2019;160(3):557-567.
57. Di Giorgio NP, Semaan SJ, Kim J, et al. Impaired GABAB receptor signaling dramatically up-regulates Kiss1 expression selectively in nonhypothalamic brain regions of adult but not prepubertal mice. *Endocrinology*. 2014;155(3):1033-1044.
58. Stephens SB, Tolson KP, Rouse ML Jr, et al. Absent progesterone signaling in Kisspeptin neurons disrupts the LH surge and impairs fertility in female mice. *Endocrinology*. 2015;156(9):3091-3097.
59. Poling MC, Luo EY, Kauffman AS. Sex differences in steroid receptor Coexpression and circadian-timed activation of Kisspeptin and RFRP-3 neurons may contribute to the sexually dimorphic basis of the LH surge. *Endocrinology*. 2017;158(10):3565-3578.
60. Mohr MA, Esparza LA, Steffen P, Micevych PE, Kauffman AS. Progesterone receptors in AVPV kisspeptin neurons are sufficient for positive feedback induction of the LH surge. *Endocrinology*. 2021;162:1-11.
61. Esparza LA, Schafer D, Ho BS, Thackray VG, Kauffman AS. Hyperactive LH pulses and elevated Kisspeptin and NKB gene expression in the arcuate nucleus of a PCOS mouse model. *Endocrinology*. 2020;161(4):1-16.
62. Castellano JM, Bentsen AH, Sanchez-Garrido MA, et al. Early metabolic programming of puberty onset: impact of changes in postnatal feeding and rearing conditions on the timing of puberty and development of the hypothalamic kisspeptin system. *Endocrinology*. 2011;152(9):3396-3408.
63. Coutinho EA, Prescott M, Hessler S, Marshall CJ, Herbison AE, Campbell RE. Activation of a classic hunger circuit slows luteinizing hormone Pulsatility. *Neuroendocrinology*. 2020;110(7–8):671-687.
64. Yang JA, Hughes JK, Parra RA, Volk KM, Kauffman AS. Stress rapidly suppresses in vivo LH pulses and increases activation of RFRP-3 neurons in male mice. *J Endocrinol*. 2018;239(3):339-350.
65. Yang JA, Song CI, Hughes JK, et al. Acute psychosocial stress inhibits LH Pulsatility and Kiss1 neuronal activation in female mice. *Endocrinology*. 2017;158(11):3716-3723.
66. Esparza LA, Terasaka T, Lawson MA, Kauffman AS. Androgen suppresses in vivo and in vitro LH pulse secretion and neural Kiss1 and Tac2 gene expression in female mice. *Endocrinology*. 2020;161(12):1-16.
67. Porteous R, Haden P, Hackwell ECR, et al. Reformulation of PULSAR for analysis of pulsatile LH secretion and a revised model of estrogen-negative feedback in mice. *Endocrinology*. 2021;162(11):1-11.
68. Dror T, Franks J, Kauffman AS. Analysis of multiple positive feedback paradigms demonstrates a complete absence of LH surges and GnRH activation in mice lacking kisspeptin signaling. *Biol Reprod*. 2013;88(6):146.
69. Steyn FJ, Wan Y, Clarkson J, Veldhuis JD, Herbison AE, Chen C. Development of a methodology for and assessment of pulsatile luteinizing hormone secretion in juvenile and adult male mice. *Endocrinology*. 2013;154(12):4939-4945.
70. Yamasaki M, Kuwahara A, Iwasa T, et al. Development-related changes in the expression of the ovarian Kiss1 and Kiss1r genes

and their sensitivity to human chorionic gonadotropin in prepubertal female rats. *J Reprod Dev*. 2017;63(4):409-414.
71. Salehi S, Adeshina I, Chen H, et al. Developmental and endocrine regulation of Kisspeptin expression in mouse Leydig cells. *Endocrinology*. 2015;156(4):1514-1522.
72. Stephens SBZ, Di Giorgio NP, Liaw RB, et al. Estradiol-dependent and -independent stimulation of Kiss1 expression in the amygdala, BNST, and lateral septum of mice. *Endocrinology*. 2018;159(9):3389-3402.
73. Kumar D, Boehm U. Genetic dissection of puberty in mice. *Exp Physiol*. 2013;98(11):1528-1534.
74. Wright H, Aylwin CF, Toro CA, Ojeda SR, Lomniczi A. Polycomb represses a gene network controlling puberty via modulation of histone demethylase Kdm6b expression. *Sci Rep*. 2021;11(1):1996.
75. Leon S, Talbi R, McCarthy EA, et al. Sex-specific pubertal and metabolic regulation of Kiss1 neurons via Nhlh2. *elife*. 2021;10:10.
76. Toro CA, Wright H, Aylwin CF, Ojeda SR, Lomniczi A. Trithorax dependent changes in chromatin landscape at enhancer and promoter regions drive female puberty. *Nat Commun*. 2018;9(1):57.
77. Lomniczi A, Wright H, Castellano JM, et al. Epigenetic regulation of puberty via zinc finger protein-mediated transcriptional repression. *Nat Commun*. 2015;6:10195.
78. Lippincott MF, Leon S, Chan YM, et al. Hypothalamic reproductive endocrine pulse generator activity independent of neurokinin B and Dynorphin signaling. *J Clin Endocrinol Metab*. 2019;104(10):4304-4318.
79. Popa SM, Moriyama RM, Caligioni CS, et al. Redundancy in Kiss1 expression safeguards reproduction in the mouse. *Endocrinology*. 2013;154(8):2784-2794.
80. Sanchez-Garrido MA, Castellano JM, Ruiz-Pino F, et al. Metabolic programming of puberty: sexually dimorphic responses to early nutritional challenges. *Endocrinology*. 2013;154(9):3387-3400.
81. Roa J, Garcia-Galiano D, Castellano JM, Gaytan F, Pinilla L, Tena-Sempere M. Metabolic control of puberty onset: new players, new mechanisms. *Mol Cell Endocrinol*. 2010;324(1–2):87-94.
82. Semaan SJ, Kauffman AS. Sexual differentiation and development of forebrain reproductive circuits. *Curr Opin Neurobiol*. 2010;20(4):424-431.
83. Hu MH, Li XF, McCausland B, et al. Relative importance of the arcuate and Anteroventral periventricular Kisspeptin neurons in control of puberty and reproductive function in female rats. *Endocrinology*. 2015;156(7):2619-2631.
84. Nandankar N, Negron AL, Wolfe A, Levine JE, Radovick S. Deficiency of arcuate nucleus Kisspeptin results in postpubertal central hypogonadism. *Am J Physiol Endocrinol Metab*. 2021;321:E264-E280.
85. Kauffman AS, Navarro VM, Kim J, Clifton DK, Steiner RA. Sex differences in the regulation of Kiss1/NKB neurons in juvenile mice: implications for the timing of puberty. *Am J Physiol Endocrinol Metab*. 2009;297(5):E1212-E1221.
86. Aerts EG, Harlow K, Griesgraber MJ, et al. Kisspeptin, neurokinin B, and Dynorphin expression during pubertal development in female sheep. *Biology (Basel)*. 2021;10(10):1-19.
87. Harlow K, Renwick AN, Shuping SL, et al. Evidence that pubertal status impacts kisspeptin/neurokinin B/dynorphin neurons in the giltdagger. *Biol Reprod*. 2021;105(6):1533-1544.
88. Lopez JA, Bedenbaugh MN, McCosh RB, et al. Does Dynorphin play a role in the onset of puberty in female sheep? *J Neuroendocrinol*. 2016;28(12):1-11.
89. Ciofi P, Lapirot OC, Tramu G. An androgen-dependent sexual dimorphism visible at puberty in the rat hypothalamus. *Neuroscience*. 2007;146(2):630-642.
90. Venancio JC, Margatho LO, Rorato R, et al. Short-term high-fat diet increases leptin activation of CART neurons and advances puberty in female mice. *Endocrinology*. 2017;158(11):3929-3942.
91. Majarune S, Nima P, Sugimoto A, et al. Ad libitum feeding triggers puberty onset associated with increases in arcuate Kiss1 and Pdyn expression in growth-retarded rats. *J Reprod Dev*. 2019;65(5):397-406.
92. Nagae M, Yamada K, Enomoto Y, et al. Conditional Oprk1-dependent Kiss1 deletion in kisspeptin neurons caused estrogen-dependent LH pulse disruption and LH surge attenuation in female rats. *Sci Rep*. 2023;13(1):20495.
93. Brockschnieder D, Lappe-Siefke C, Goebbels S, Boesl MR, Nave KA, Riethmacher D. Cell depletion due to diphtheria toxin fragment a after Cre-mediated recombination. *Mol Cell Biol*. 2004;24(17):7636-7642.
94. Jung S, Unutmaz D, Wong P, et al. In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. *Immunity*. 2002;17(2):211-220.
95. Bennett CL, van Rijn E, Jung S, et al. Inducible ablation of mouse Langerhans cells diminishes but fails to abrogate contact hypersensitivity. *J Cell Biol*. 2005;169(4):569-576.
96. Brousse B, Mercier O, Magalon K, et al. Characterization of a new mouse line triggering transient oligodendrocyte progenitor depletion. *Sci Rep*. 2023;13(1):21959.
97. Wu SY, Chiang CS. Distinct role of CD11b(+)Ly6G(−)Ly6C(−) myeloid-derived cells on the progression of the primary tumor and therapy-associated recurrent brain tumor. *Cells*. 2019;9(1):1-17.
98. Saito M, Iwawaki T, Taya C, et al. Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice. *Nat Biotechnol*. 2001;19(8):746-750.
99. Kisler K, Nikolakopoulou AM, Sweeney MD, Lazic D, Zhao Z, Zlokovic BV. Acute ablation of cortical Pericytes leads to rapid neurovascular uncoupling. *Front Cell Neurosci*. 2020;14:27.
100. Bruttger J, Karram K, Wortge S, et al. Genetic cell ablation reveals clusters of local self-renewing microglia in the mammalian central nervous system. *Immunity*. 2015;43(1):92-106.
101. Luquet S, Perez FA, Hnasko TS, Palmiter RD. NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates. *Science*. 2005;310(5748):683-685.
102. Nagae M, Uenoyama Y, Okamoto S, et al. Direct evidence that KNDy neurons maintain gonadotropin pulses and folliculogenesis as the GnRH pulse generator. *Proc Natl Acad Sci USA*. 2021;118(5):1-11.
103. Clarkson J, d'Anglemont de Tassigny X, Moreno AS, Colledge WH, Herbison AE. Kisspeptin–GPR54 signaling is essential for Preovulatory gonadotropin-releasing hormone neuron

activation and the luteinizing hormone surge. *J Neurosci*. 2008; 28(35):8691-8697.

104. Robertson JL, Clifton DK, de la Iglesia HO, Steiner RA, Kauffman AS. Circadian regulation of Kiss1 neurons: implications for timing the Preovulatory GnRH/LH surge. *Endocrinology*. 2009;150:3664-3671.
105. Stincic TL, Qiu J, Connors AM, Kelly MJ, Ronnekleiv OK. Arcuate and preoptic Kisspeptin neurons exhibit differential projections to hypothalamic nuclei and exert opposite postsynaptic effects on hypothalamic paraventricular and dorsomedial nuclei in the female mouse. *eNeuro*. 2021;8(4):1-26.
106. Yeo SH, Kyle V, Morris PG, et al. Visualisation of Kiss1 Neurone distribution using a Kiss1-CRE transgenic mouse. *J Neuroendocrinol*. 2016;28(11):1-12.
107. Lin XH, Lass G, Kong LS, et al. Optogenetic activation of arcuate Kisspeptin neurons generates a luteinizing hormone surge-like secretion in an estradiol-dependent manner. *Front Endocrinol (Lausanne)*. 2021;12:775233.

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