

Acute Suppression of LH Secretion by Prolactin in Female Mice Is Mediated by Kisspeptin Neurons in the Arcuate Nucleus

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Hyperprolactinemia causes infertility, but the specific mechanism is unknown. It is clear that elevated prolactin levels suppress pulsatile release of GnRH from the hypothalamus, with a consequent reduction in pulsatile LH secretion from the pituitary. Only a few GnRH neurons express prolactin receptors (Prlrs), however, and thus prolactin must act indirectly in the underlying neural circuitry. Here, we have tested the hypothesis that prolactin-induced inhibition of LH secretion is mediated by kisspeptin neurons, which provide major excitatory inputs to GnRH neurons. To evaluate pulsatile LH secretion, we collected serial blood samples from diestrous mice and measured LH levels by ultrasensitive ELISA. Acute prolactin administration decreased LH pulses in wild-type mice. Kisspeptin neurons in the arcuate nucleus and in the rostral periventricular area of the third ventricle (RP3V) acutely responded to prolactin, but prolactin-induced signaling in kisspeptin neurons was up to fourfold higher in the arcuate nucleus when compared with the RP3V. Consistent with this, conditional knockout of Prlr specifically in arcuate nucleus kisspeptin neurons prevented prolactin-induced suppression of LH secretion. Our data establish that during hyperprolactinemia, suppression of pulsatile LH secretion is mediated by Prlr on arcuate kisspeptin neurons. (*Endocrinology* 160: 1323–1332, 2019)

Hyperprolactinemia is a major cause of infertility in men and women (1–4) and is associated with a reduction in the pulsatile secretion of LH (5, 6). Although hyperprolactinemia typically is treated using a dopamine agonist to suppress prolactin secretion (7, 8), in the absence of such treatment, fertility can be restored through the pulsatile administration of GnRH (5, 9, 10). This suggests that a hypothalamic deficit is the primary cause of hyperprolactinemia-induced infertility, and potential adverse actions of prolactin in the pituitary (11, 12) and/or gonad (13–15) are not insurmountable. Although numerous animal models have confirmed that chronic elevations in circulating prolactin levels cause the

reduced frequency and amplitude of LH pulses (16–19), the underlying mechanism of prolactin action on GnRH neurons has remained elusive. We have shown that the majority of GnRH neurons do not express the prolactin receptor (Prlr) (20–22), suggesting that prolactin action must be mediated indirectly, through a prolactin-sensitive afferent input (21).

Since first being identified ~15 years ago as essential for puberty in humans (23, 24), kisspeptin neurons have emerged as being a critical afferent regulator of GnRH neurons. These neurons are found in two major populations, one in the rostral periventricular area of the third ventricle (RP3V), which appears to be essential for

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Abbreviations: Arc, arcuate nucleus; AVPV, anteroventral periventricular nucleus; cPVpo, caudal periventricular preoptic area; DIG, digoxigenin; eGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; Prlr, prolactin receptor; pSTAT5, phosphorylation of signal transducer and activator of transcription 5; RP3V, rostral periventricular area of the third ventricle; rPVpo, rostral periventricular preoptic area; SSC, saline sodium citrate.

ovulation, and another in the arcuate nucleus (Arc) of the hypothalamus, which seems to be involved in the negative feedback regulation of GnRH secretion. Recent evidence suggests that the Arc population of kisspeptin neurons, in particular, form the intrinsic “pulse generator” that governs minute-to-minute activity of GnRH neurons (25). We have shown that both populations of kisspeptin neurons express *Prlr* and respond to prolactin action with an increase in phosphorylation of signal transducer and activator of transcription 5 (pSTAT5) (21, 26). Expression of kisspeptin in both populations in the mouse is reduced when prolactin levels are high (26–28). On the other hand, in the rat, prolactin inhibition is more prominent in the arcuate population (29). Collectively, these data raise the possibility that prolactin exerts its effect on fertility through suppression of the kisspeptin inputs to GnRH neurons, although kisspeptin neurons in the RP3V and Arc may subserve differential roles in this effect of prolactin. Consistent with this concept, administration of kisspeptin has been reported to restore ovarian function in a mouse model of hyperprolactinemia (27) and to increase LH secretion in women with hyperprolactinemia-induced amenorrhea (30). Although these data could be interpreted to suggest that prolactin causes a deficit in afferent kisspeptin inputs to GnRH neurons, it is also possible that because kisspeptin is such a powerful secretagogue for GnRH, exogenous kisspeptin could overcome any inhibitory input onto GnRH neurons. Thus, it remains to be determined whether suppression of kisspeptin secretion is necessary or sufficient to account for the prolactin-induced suppression of pulsatile GnRH secretion. Here, we have directly tested this hypothesis, using a conditional knockout approach that specifically removed *Prlr* from the arcuate population of kisspeptin neurons in mice.

Methods

Animals

Adult, wild-type, female C57BL/6J mice and heterozygous Kiss1-Cre (31) mice were crossed with Ai9 Cre-dependent tdTomato reporter mice [B6.Cg-Gt(ROSA)26Sor^{tm9(CAG-tdTomato)Hze/J}; Jackson ImmunoResearch Laboratories, West Grove, PA] (32) or *Prlr*^{lox/lox} mice (33). All mice were used as adults (10 to 16 weeks old) and group housed under conditions of controlled temperature (22°C ± 1°C) and lighting (12-hour light and 12-hour dark cycles, with lights on at 7:00 AM) with *ad libitum* access to food and water. All animal experimental protocols were approved by the University of Otago animal ethics committee.

Evaluation of pulsatile LH secretion

Groups of C57BL/6J (*n* = 5 to 6), *Prlr*^{lox/lox} (*n* = 9 to 10), and *Prlr*^{lox/lox}/Kiss1-Cre (*n* = 7 to 8) female mice were habituated to handling daily for 4 weeks, and the estrous cycle was

monitored by daily collection of vaginal smears. On the morning of diestrus, ovine prolactin (5 mg/kg subcutaneous injection) or vehicle (saline) was administered to mice 120 minutes before blood sampling. Blood sampling and measurement of LH by ELISA was undertaken as reported in the literature (34–38). LH secretion was measured by obtaining 4-μL blood samples every 6 minutes from the tail tip over 120 minutes. Blood sampling was repeated 2 weeks later, with mice receiving the alternative treatment to what they received in the first bleeding experiment. Whole-blood samples were diluted 1:13 for measurement of LH.

The assay sensitivity was 0.002 ng/mL to 4 ng/mL, with an intra-assay coefficient of variation of 3.90% and interassay coefficient of variation of 6.25%. A pulse of LH was conservatively defined as a peak level of LH >20% above the preceding value. For each detected pulse, the amplitude was determined by subtracting the basal LH concentration immediately before the pulse from the maximum LH concentration of the pulse. Mean LH levels were calculated by averaging all the LH levels collected during the 120-minute experiment. For each LH parameter investigated, the mean ± SEM for each group was calculated and compared by two-way ANOVA with uncorrected Fisher least significant difference tests performed to detect significance between groups, where *P* < 0.05 was considered a statistically significant different result.

Immunohistochemistry

To evaluate the proportion of kisspeptin neurons that respond to prolactin, virgin female Kiss1-Cre/tdTomato mice (*n* = 5) were injected with ovine prolactin (5 mg/kg IP injection; Sigma-Aldrich, St. Louis, MO), anesthetized with sodium pentobarbital, and 45 minutes later were transcardially perfused with 4% paraformaldehyde. Brains were removed, postfixed in the same solution, and cryoprotected in 30% sucrose overnight. Two sets of 30-μm-thick coronal sections through the forebrain from each animal were cut on a sliding microtome. One series of tissue was used to examine pSTAT5 labeling (39) as previously described (40), with the addition of tyramide amplification to allow sensitive detection of pSTAT5 by fluorescent immunohistochemistry. Briefly, after antigen retrieval, sections were incubated in rabbit anti-pSTAT5 primary antibody (pSTAT5 Tyr694, 1:800; Cell Signaling Technology, Beverly, MA) for 72 hours at 4°C, followed by a 60-minute incubation in biotinylated goat anti-rabbit IgG [1:200; Vector Laboratories, Peterborough, United Kingdom (41)]. Sections were then incubated in Vector Elite avidin-biotin-horseradish peroxidase complex (1:100) for 45 minutes before being incubated in Biotin-XX Tyramide (0.3%; Invitrogen, Carlsbad, CA). Finally, sections were incubated in a Streptavidin 647 IgG [1:400; AlexaFluor; Invitrogen (42)] for 2 hours at 37°C. Images were collected using a Nikon A1 inverted confocal and ×20 objective. Z stacks were collected with images taken 1.2-μm apart.

Quantification of the proportion of kisspeptin neurons expressing pSTAT5 was undertaken by counting the total number of kisspeptin neurons (endogenous cre-dependent tdTomato fluorescence driven by the Kiss1 promoter), and the total number of kisspeptin neurons colabeled with pSTAT5 in two sections per animal in three levels of the RP3V; the anteroventral periventricular nucleus (AVPV), rostral periventricular preoptic area (rPVpo), caudal periventricular preoptic area (cPVpo), and three levels of the Arc: rostral, mid, and

caudal. Bregma coordinates for the RP3V were 0.38, 0.14, and 0.02 (AVPV, rPVpo and cPVpo, respectively) and for the Arc were -1.34, -1.70, and -2.18 (rostral, mid, and caudal Arc, respectively).

To evaluate *Prlr* expression in kisspeptin neurons in *Prlr*^{lox/lox}/Kiss1-Cre mice, brains were processed for the presence of enhanced green fluorescent protein (eGFP) immunoreactivity [which indicated Cre-dependent recombination in *Prlr*^{lox/lox}/Kiss1-Cre mice (33)]. Brains from groups of *Prlr*^{lox/lox}/Kiss1-Cre and control *Prlr*^{lox/lox} mice ($n = 6$) were collected and sectioned as described earlier in this section and one series of tissue was used to examine eGFP expression (43) by chromagen immunohistochemistry, as previously described (33). No eGFP expression was observed at any level of the RP3V; therefore, this region was not subdivided in this experiment. Quantification of GFP labeling was undertaken using a $\times 20$ objective by counting the total number of eGFP-labeled cells in two sections at each level of the rostral, mid, and caudal arc. The mean (\pm SEM) number of eGFP labeled cells per 30- μ m section was calculated and differences between control *Prlr*^{lox/lox} and kisspeptin neuron-specific *Prlr* KO (*Prlr*^{lox/lox}/Kiss1-Cre) mice were compared by two-way ANOVA. Statistically significant differences ($P < 0.05$) were identified by a Tukey *post hoc* test.

Double-label *Prlr*/Kiss-1 in situ hybridization histochemistry

Groups of *Prlr*^{lox/lox} and *Prlr*^{lox/lox}/Kiss1-Cre mice underwent bilateral ovariectomy at 13 to 15 weeks ($n = 6$). Ovaries were removed from mice under anesthesia with isoflurane through dorsal lateral incisions. Two weeks post-surgery, half of each group also received a subcutaneous 17 β -estradiol implant, 4 days before brain collection. The implants were made by combining 17 β -estradiol (1 mg/mL; Sigma-Aldrich) in 100% (v/v) ethanol with Silastic Medical grade A silicone adhesive (Dow Corning, Midland, MI) to achieve a 0.1-mg/mL concentration, which was loaded into Silastic tubing (internal diameter, 1.02 mm; external diameter, 2.16 mm; Dow Corning), with each implant achieving a dose of 5 μ g/kg estradiol. This results in low serum estradiol concentrations of 4 to 7 pg/mL (44). Mice were transcardially perfused with 2% paraformaldehyde while under sodium pentobarbital anesthesia. Brains were dissected out and postfixed in the same fixative for 1 to 2 hours before being cryoprotected in 30% sucrose overnight. Coronal sections (14- μ m thick) were cut in a cryostat and float mounted in milli-Q water onto Superfrost Plus slides. Sections were air dried, then stored at -20°C until use.

mRNA sequences from Genbank were used to design primer sets in PrimerBLAST for *Prlr* (accession no. NM_011169; nucleotides 1530 to 1710) and *Kiss-1* (accession no. NM_178260.3; nucleotides 93 to 306). Primer pairs with T7 and SP6 polymerase promoter sequences incorporated on the 5' ends were used to generate DNA templates by PCR. Template specificity was checked by Sanger sequencing. Antisense and sense [35-S] UTP-labeled and digoxigenin (DIG)-UTP labeled riboprobes were generated using an *in vitro* transcription kit (Promega, Madison, WI) and DIG RNA labeling kit SP6/T7 (Roche, Mannheim, Germany) in accordance with manufacturer's instruction. Resultant probes were filtered to remove unincorporated nucleotides or DIG using Mini quick-spin RNA columns (Roche, Indianapolis, IN).

Double-label *in situ* hybridization histochemistry was performed as previously described (45). Briefly, sections were postfixed in 2% paraformaldehyde in 0.1 M phosphate buffer, washed in 0.5 \times saline sodium citrate (SSC; 75 nmol/L sodium chloride, 7.5 mmol/L trisodium citrate dihydrate) and tissue permeabilized in proteinase K (2 μ g/mL; Roche, Mannheim, Germany). Tissue was acetylated in 0.1 M triethanolamine containing 0.25% (v/v) acetic anhydride, before prehybridization in an ethanol series and chloroform immersion. Probes were denatured and combined with ice-cold hybridization buffer [50% (v/v) formamide, 0.3 mol/L sodium chloride, 1 \times Denhardt solution, 20 mmol/L Tris, 5 mmol/L EDTA, 10% (w/v) dextran sulfate, and 100 mmol/L dithiothreitol]. The probe mixture containing probes at concentrations of 0.9×10^6 cpm/90 μ L ([35-S]-UTP labeled *Prlr* riboprobe) and 50 ng/120 μ L (*Kiss-1* DIG-labeled probe) was pipetted onto each slide. Sections were hybridized at 55°C overnight. After hybridization, sections were washed in 2 \times SSC, treated with RNase A (20 μ g/L), then left in a prolonged 2-hour wash in 2 \times SSC with 10 mmol/L β -mercaptoethanol and 1 mmol/L EDTA at 65°C.

For DIG detection, sections were treated with anti-DIG alkaline phosphatase fab fragments (1:2000) followed by levamisole (1 mg/mL). Alkaline phosphatase activity was detected on sections using 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt solution. The reaction was stopped after 8 hours with four 30-minute washes in phosphate/saline/EDTA buffer. Sections were briefly dipped in distilled water and 70% (v/v) ethanol, then air dried overnight.

To visualize the *Prlr* [35-S]-UTP labeled riboprobe, sections were coated with K.5D nuclear emulsion (Harman Technology, Cheshire, United Kingdom) and stored in light-safe containers at 4°C for 8 weeks. Sections were developed with Kodak D19 developer and 30% (w/v) sodium thiosulfate as fixer, then dried at 42°C, immersed in two changes of xylene, and cover slipped with Vectamount (Vector Laboratories, Burlingame, CA).

Quantification of the proportion of kisspeptin neurons coexpressing *Prlr* mRNA was undertaken on an Olympus AX70 using a $\times 40$ objective, by counting the total number of *kiss1*-labeled cells and the total number that showed *Prlr* mRNA expression. A *kiss1*-labeled cell was classified as coexpressing *Prlr* mRNA if the density of *Prlr* silver grains was five times that of background. The mean (\pm SEM) percentage of Kiss1 cells coexpressing *Prlr* mRNA was calculated per group and differences between control *Prlr*^{lox/lox} and *Prlr*^{lox/lox}/Kiss1-Cre mice was determined by Student *t* tests, where $P < 0.05$ was considered a statistically significant difference.

Results

Prolactin acutely suppressed the pulsatile secretion of LH in mice

We first aimed to investigate whether administration of prolactin could acutely alter the pulsatile pattern of LH secretion in diestrous C57Bl/6J female mice. Vehicle-treated female mice showed a pulsatile pattern of LH secretion, with, on average, 3.2 ± 0.58 pulses of LH secretion over 120 minutes (Fig. 1), consistent with previous reports (35). After acute prolactin administration, however, the LH pulse frequency was significantly suppressed to 1.0 ± 0.58 pulses in 120 minutes ($P =$

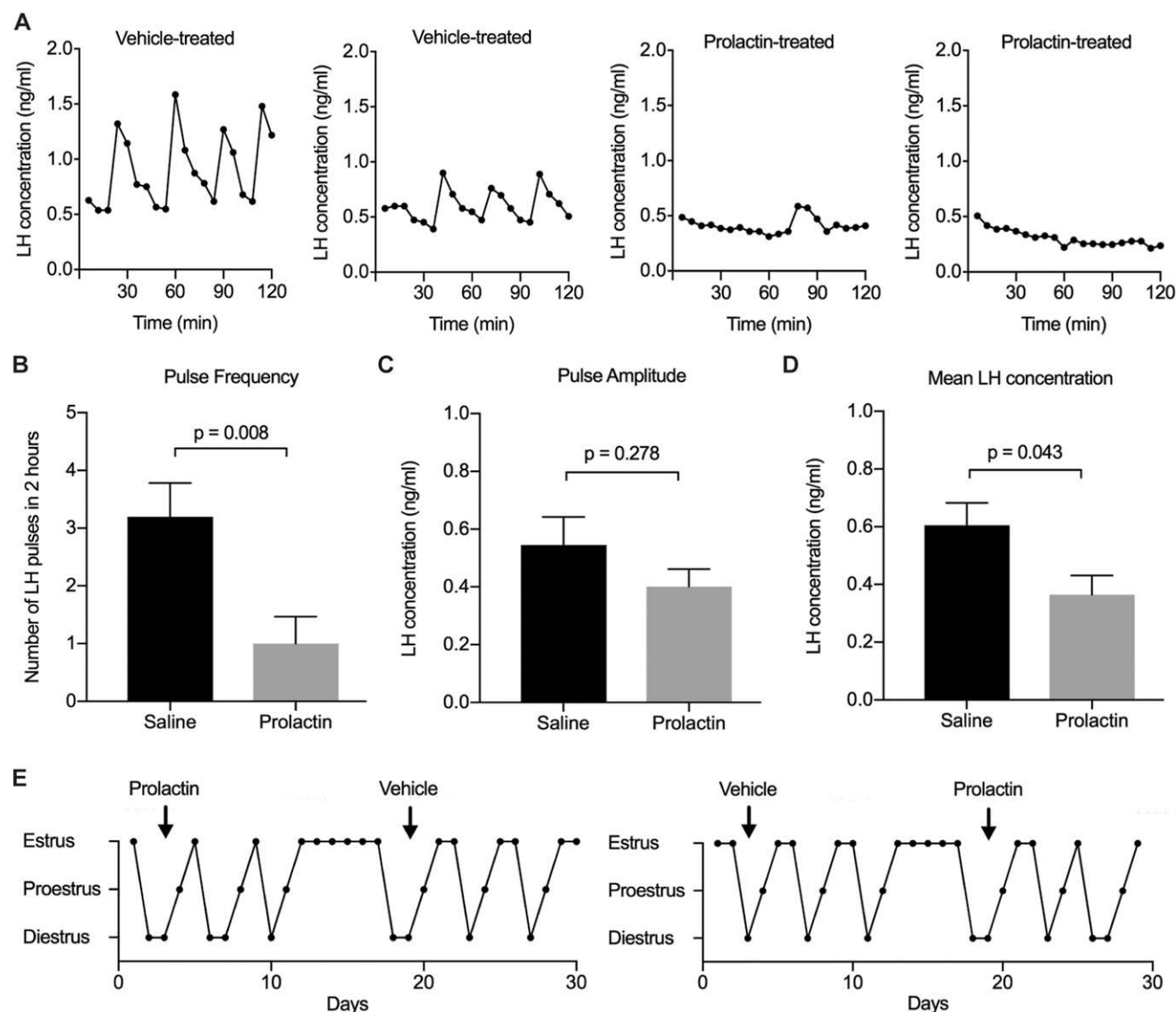


Figure 1. Suppression of pulsatile LH secretion after acute prolactin administration in wild-type, gonadally intact C57Bl/6J female mice. (A) Representative examples of pulsatile LH secretion in two vehicle-treated and two prolactin-treated mice. (B, D) Group mean (\pm SEM) number of LH pulses and mean LH concentration were suppressed after acute prolactin administration, whereas (C) there was no change in the mean (\pm SEM) amplitude of each pulse ($n = 5$ to 6). (E) The pattern of vaginal smears observed in two representative mice, showing there was no effect of acute prolactin administration on estrous cyclicity.

0.008), accompanied by a significant reduction in mean LH concentration ($P = 0.043$) but not in pulse amplitude (Fig. 1B–1D). In contrast to the suppression of estrous cycles (persistent diestrus or pseudopregnancy) induced by chronic prolactin administration (27), acute prolactin administration on diestrus did not disrupt estrous cyclicity, with all mice entering proestrus 24 hour after prolactin administration (Fig. 1E).

Prolactin induced prolactin receptor-mediated signal transduction in kisspeptin neurons in the RP3V and Arc

To assess the prolactin-responsiveness of kisspeptin neurons in gonadally intact mice, we next generated mice with tdTomato expression targeted to kisspeptin neurons

[using a Kiss1-Cre (31)]. This was particularly important because previous work examining prolactin responses in kisspeptin neurons has been compromised by the fact that arcuate kisspeptin neurons are not readily detected in the presence of estradiol and usually require ovariectomy for visualization (46). Prolactin-induced pSTAT5 was used as a marker of Prlr-mediated signal transduction (40). We have previously demonstrated that bromocriptine treatment to block endogenous prolactin results in the absence of pSTAT5 immunoreactivity in kisspeptin neurons and only low levels of pSTAT5 in kisspeptin neurons of vehicle-treated mice (26). In the RP3V of female Kiss1-Cre/tdTomato mice, $38.5\% \pm 6.7\%$ of AVPV tdTomato-expressing cells showed prolactin-induced pSTAT5, whereas few periventricular

preoptic kisspeptin neurons responded to prolactin ($9.6\% \pm 3.4\%$ and $7.0\% \pm 2.8\%$ in the rostral and caudal periventricular preoptic, respectively; Fig. 2). In the AVPV, 126.2 ± 24.03 tdTomato-expressing cells were counted per 30- μm section, 131.6 ± 17.11 in the rPVpo and 107.2 ± 14.12 in the cPVpo. Significantly higher proportions of kisspeptin neurons in the Arc were prolactin responsive, with $70.6\% \pm 5.9\%$ of kisspeptin neurons showing prolactin-induced pSTAT5 across all levels of the Arc (Fig. 2). The mean number of tdTomato-expressing cells counted per 30- μm section was $75.88 \pm$

7.84 , 142.9 ± 15.72 , and 172.1 ± 8.80 at the level of the rostral, mid, and caudal arcuate, respectively.

Arcuate kisspeptin neurons directly mediated the suppression of LH pulse frequency by prolactin

To investigate whether kisspeptin neurons mediate the action of prolactin in suppressing the pulsatile release of LH, we generated mice in which *Prlr* was conditionally deleted from kisspeptin neurons (*Prlr*^{lox/lox}/*Kiss1*-Cre), using a *Prlr*^{lox/lox} allele (33, 47) crossed with a *Kiss1*-IRES-Cre mouse line (31). Unexpectedly, this genetic

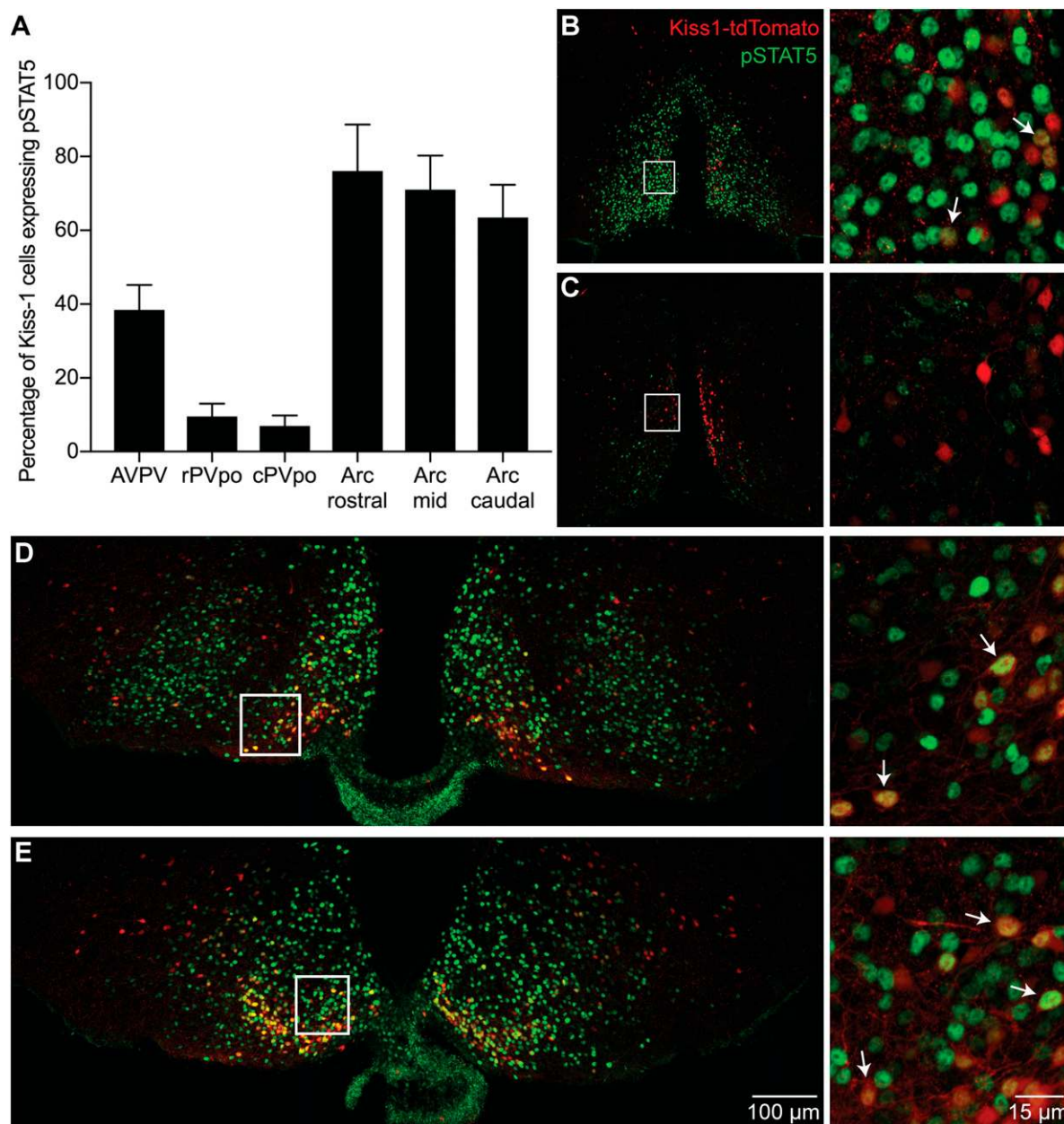


Figure 2. Functional prolactin responses in kisspeptin neurons in the RP3V and Arc in gonadally intact female *Kiss1*-Cre/tdTomato mice. The percentage of kisspeptin neurons colabeled with prolactin-induced pSTAT5 are shown in (A) ($n = 5$). Photomicrographs show immunohistochemical labeling of pSTAT5 (green nuclear labeling) in kisspeptin cells (identified by endogenous tdTomato expression in red) in the (B) anteroventral periventricular nucleus (AVPV), (C) rostral periventricular preoptic area (rPVpo), (D and E) mid and caudal Arc of *Kiss1*-Cre/tdTomato mice. Labeling was also quantified in the cPVpo (not illustrated). White arrows indicate kisspeptin cells (red) double labeled for pSTAT5 (green).

cross resulted in an arcuate-specific knockdown of *Prlr* but did not affect the RP3V kisspeptin neuron population (Fig. 3). The *Prlr*^{lox/lox} allele has been designed such that Cre-mediated recombination of the *Prlr* gene results in an inversion of the targeted sequence, eliminating the *Prlr* after exon 5 and inserting a correctly orientated copy of the sequence for eGFP in place, with expression of green fluorescent protein (GFP), therefore, driven by the *Prlr* promoter, providing a marker for neurons in which this receptor has been deleted (33). Immunolabeling for GFP revealed that successful recombination of the *Prlr* gene occurred throughout the Arc but not in the RP3V of female *Prlr*^{lox/lox}/Kiss1-Cre mice (Fig. 3A and 3B). No GFP immunolabeling was observed in the amygdala, where expression of *Kiss-1* mRNA has previously been reported in mice and rats (48).

To confirm the arcuate-specific nature of the deletion, *in situ* hybridization was used to colabel *Prlr* and *Kiss-1* mRNA. To ensure we could detect *Kiss-1* mRNA in the different populations, animals were ovariectomized, with half of them treated with 17 β -estradiol for visualization of *Kiss-1* mRNA in the RP3V and the rest untreated to detect *Kiss-1* mRNA in the Arc (46). In control *Prlr*^{lox/lox} mice, the presence of *Prlr* mRNA was confirmed in both populations of *Kiss-1* neurons. Although *Prlr* mRNA was markedly suppressed in the Arc of *Prlr*^{lox/lox}/Kiss1-Cre mice (92.4% \pm 1.6% of *Kiss-1* neurons in the Arc coexpressed *Prlr* mRNA in control *Prlr*^{lox/lox} mice compared with 21.7% \pm 4.5% in *Prlr*^{lox/lox}/Kiss1-Cre mice; $P < 0.0001$), in the RP3V, there was no reduction in the proportion of RP3V *Kiss-1* cells expressing *Prlr* mRNA (56.9% \pm 3.8% in control *Prlr*^{lox/lox} mice compared with 61.9% \pm 2.8% in *Prlr*^{lox/lox}/Kiss1-Cre mice; $P = 0.355$; Fig. 3C and 3D). There was no difference in the number of cells expressing *Kiss-1* mRNA between *Prlr*^{lox/lox} and *Prlr*^{lox/lox}/Kiss1-Cre mice in either the RP3V (67.5 \pm 3.5 cells in *Prlr*^{lox/lox} mice compared with 40.0 \pm 9.2 cells in *Prlr*^{lox/lox}/Kiss1-Cre mice; $P < 0.12$) or the Arc (106.0 \pm 27.5 cells in *Prlr*^{lox/lox} mice compared with 81.7 \pm 18.4 cells in *Prlr*^{lox/lox}/Kiss1-Cre mice; $P < 0.529$).

Prolactin-induced suppression of the pulsatile secretion of LH was investigated in mice with an arcuate-specific knockout of *Prlr*. As seen in wild-type mice, in control *Prlr*^{lox/lox} mice, acute subcutaneous prolactin administration significantly suppressed the frequency of LH pulses from 3.72 \pm 0.63 pulses in 120 minutes in control mice to 1.95 \pm 0.49 pulses in *Prlr*^{lox/lox}/Kiss1-Cre mice ($P = 0.018$; Fig. 4). Acute prolactin administration did not suppress the frequency of LH in arcuate kisspeptin-specific *Prlr* knockdown mice, with 3.75 \pm 0.62 pulses in control mice and 3.86 \pm 0.55 pulses in *Prlr*^{lox/lox}/Kiss1-Cre mice ($P = 0.450$; Fig. 4C). There was no difference in pulse amplitude between groups (Fig.

4D); however, mean LH concentration was significantly suppressed in control mice ($P = 0.038$) but not in *Prlr*^{lox/lox}/Kiss1-Cre mice ($P = 0.832$; Fig. 4E). As seen in wild-type C57Bl/6J mice, acute prolactin administration did not disrupt estrous cyclicity in either control or arcuate-specific *Prlr* knockdown mice (Fig. 4F). Taken together, our data demonstrate that the prolactin-induced suppression of pulsatile release of LH is specifically mediated by kisspeptin neurons in the Arc and provide a mechanism for hyperprolactinemia-induced infertility.

Discussion

Our data establish the arcuate kisspeptin neurons as being a key intermediary of the role of prolactin in acute suppression of pulsatile LH secretion. Although arcuate and RP3V kisspeptin neurons express *Prlr*, a much higher proportion of arcuate neurons appear to be prolactin responsive. Moreover, our data demonstrate that specific deletion of *Prlr* on Arc kisspeptin cells is sufficient to prevent the ability of prolactin to acutely suppress pulsatile LH secretion. Continued expression of *Prlr* on RP3V kisspeptin cells is unable to compensate for the absence of *Prlr* in the arcuate population.

The expression of the *Prlr* in the majority of arcuate kisspeptin neurons and in approximately half of the RP3V kisspeptin neurons is consistent with findings of previous studies (21, 26, 29). The use of a tdTomato reporter line, driven by the Kiss1-cre promoter, allowed the investigation of prolactin sensitivity of RP3V and arcuate populations in gonadally intact female mice, with the poor visualization of arcuate kisspeptin neuron cell bodies in gonadally intact rodents being a limiting factor in previous studies. In this model, the proportion of kisspeptin neurons in the arcuate that were responsive to prolactin was almost four times that of the RP3V population. It remains possible, however, that the proportion of cells responding in the RP3V might be sensitive to estradiol, with low levels present in the diestrous animals used for this study.

One concern was that we may have effectively knocked out *Prlr* in the RP3V, but that levels of GFP driven off the *Prlr* promoter in these kisspeptin neurons were too low to detect. However, the *in situ* hybridization experiment demonstrated an unchanged number of *Kiss-1* neurons expressing *Prlr* mRNA in the RP3V population, whereas *Prlr* mRNA was clearly eliminated in the majority of the arcuate *Kiss-1* cells. Reasons for failure in the *Prlr*^{lox/lox}/Kiss1-Cre mice to knock out *Prlr* in RP3V, when this Cre line clearly drove recombination in the reporter line and in other previous studies (31, 49), are unclear. Recombination of a floxed allele depends on at least two factors: the level of Cre expression and the

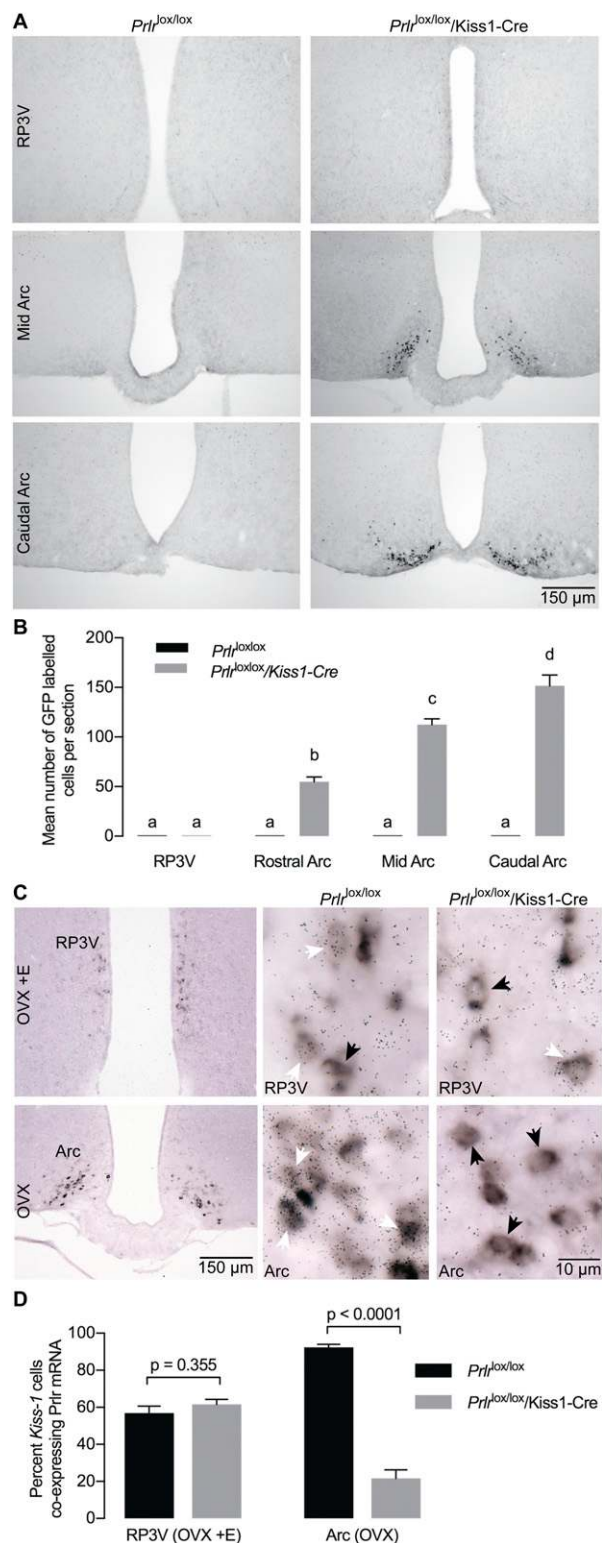


Figure 3. Conditional deletion of the *Prlr* from arcuate kisspeptin neurons. (A) Immunohistochemical labeling for eGFP indicating successful recombination of the *Prlr* gene in the Arc but not in the RP3V of kisspeptin-neuron-specific prolactin receptor knockout (*Prlr^{lox/lox}/Kiss1-Cre*) mice. (B) Quantification of group means ($n = 6$). No eGFP labeling was present in control (*Prlr^{lox/lox}*) mice. (C, D) *In situ* hybridization for *Prlr* and *Kiss-1* mRNA showed no change in the mean (\pm SEM) proportion of kisspeptin neurons expressing *Prlr* mRNA in the RP3V of OVX + E *Prlr^{lox/lox}/Kiss1-Cre* mice ($n = 3$ to 4). In contrast, there was a marked decrease in the number of *Kiss-1*

accessibility of the locus to be recombined. Accessibility may be affected by factors such as the chromatin structure at any particular individual locus. The R26 locus harboring the tdTomato reporter will have a different accessibility than any other locus in the genome, so it cannot be expected to exactly report where a different floxed gene will be recombined. It is possible that high levels of Cre could compensate for low accessibility. Although internal ribosome entry site constructs typically target highly accurate expression to a cell population (50), levels of genes driven by the internal ribosome entry site construct are typically found at significantly lower levels than the targeted gene (51–53). Our experience with this particular *Prlr^{lox/lox}* line is that high levels of Cre are required to drive recombination, with some Cre lines not causing effective recombination of the *Prlr* gene when it would be expected. For example, although we have shown recombination of the *Prlr^{lox/lox}* construct in tuberoinfundibular dopamine neurons using a calcium calmodulin kinase IIa-Cre (33), a dopamine transporter Cre that can induce various reporter molecules in these cells (54) does not drive recombination of *Prlr^{lox/lox}*. This is likely due to low expression of dopamine transporter in this particular population of dopaminergic neurons (55, 56). In addition, the time of expression of Cre might also be important. Using these Kiss1-Cre mice, Cre expression is initiated exclusively in the arcuate kisspeptin population at approximately embryonic day 13.5 and persists throughout development, whereas expression in the RP3V starts weeks later and only peaks at puberty (57, 58).

The unexpected restriction of Cre-mediated recombination of the *Prlr^{lox}* allele to the Arc was serendipitous because it enabled us to specifically evaluate the role of this kisspeptin population in mediating the acute effect of prolactin on pulsatile secretion of LH. With recent *in vivo* recording of the activity of arcuate kisspeptin cells providing compelling evidence that they form the intrinsic pulse generator for GnRH neurons (25), these neurons provide a potential conduit for mediating the suppression of LH pulsatile secretion by prolactin. The loss of prolactin-induced suppression of the pulsatile pattern of LH secretion in mice with arcuate kisspeptin neuron-specific deletion of the *Prlr* established that acute prolactin action on GnRH pulses is mediated by this population of cells. It remains possible that other populations of prolactin-sensitive neurons in the hypothalamus might contribute to

Figure 3. (Continued). neurons expressing *Prlr* mRNA in the Arc of OVX *Prlr^{lox/lox}/Kiss1-Cre* mice compared with control *Prlr^{lox/lox}* mice. Different letters represent statistically different groups ($P < 0.001$). White arrows indicate neurons double labeled for *Prlr* and *Kiss1* mRNA. Black arrows indicate neurons single labeled for *Kiss1* mRNA. OVX, ovariectomized; OVX + E, ovariectomized and estradiol treated.

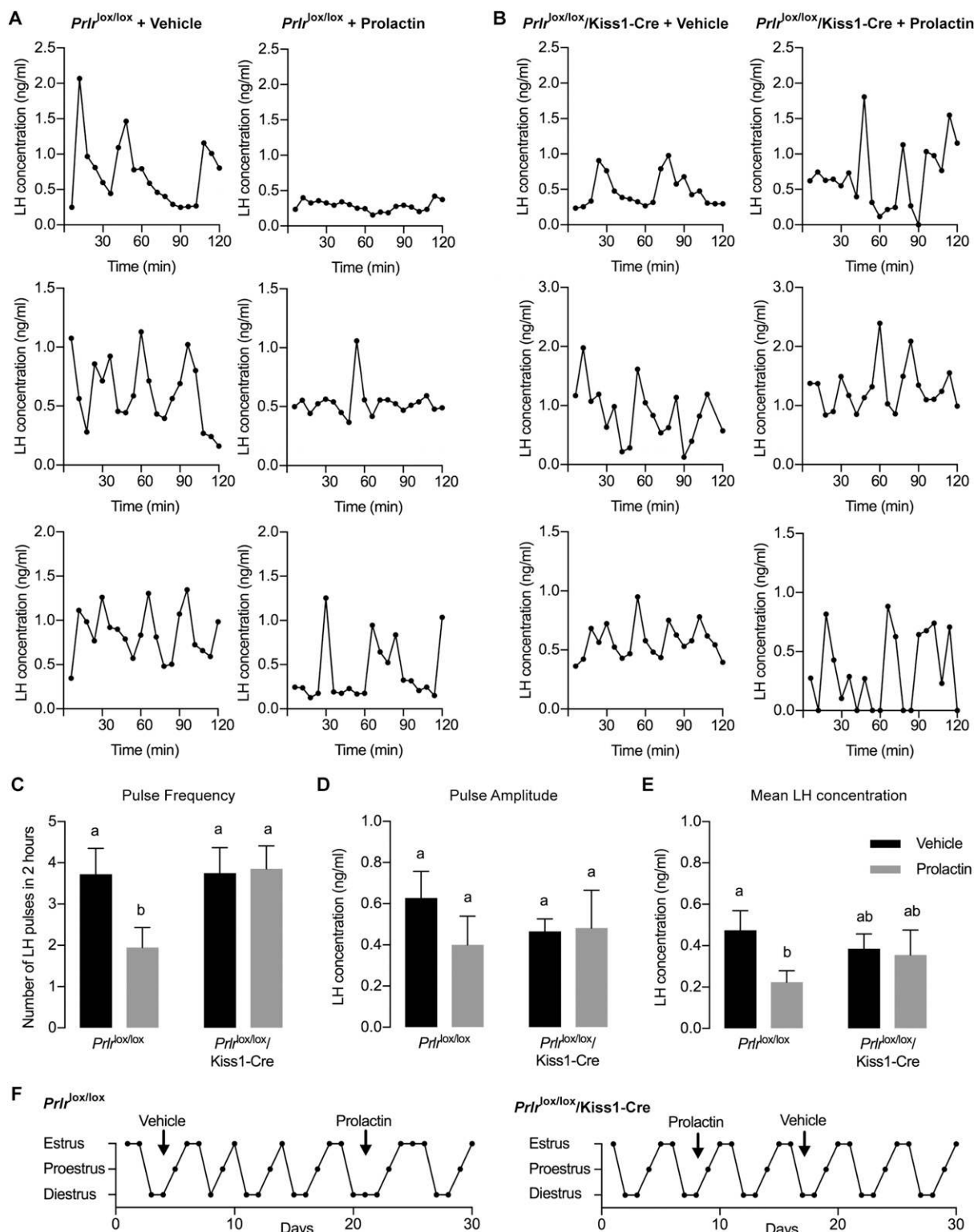


Figure 4. Arcuate-specific deletion of *Prlr* from kisspeptin neurons prevents the prolactin-induced suppression of LH pulsatile secretion. (A, B) Representative examples of pulsatile LH secretion in three control (*Prlr^{lox/lox}*) and three kisspeptin neuron-specific *Prlr* knockout (*Prlr^{lox/lox}/Kiss1-Cre*) mice after vehicle and prolactin treatment, respectively. (B) Note the presence of pulsatile LH secretion after acute subcutaneous prolactin administration in *Prlr^{lox/lox}/Kiss1-Cre* mice. (C, E) The mean (\pm SEM) number of LH pulses and mean LH concentration were suppressed after acute prolactin administration in control but not in *Prlr^{lox/lox}/Kiss1-Cre* mice ($n = 7$ to 10). (D) The mean (\pm SEM) amplitude of each pulse was not affected by acute prolactin treatment in either group. Different letters represent statistically different groups ($P < 0.05$). (F) There was no effect of acute prolactin administration on estrous cyclicity in either control or *Prlr^{lox/lox}/Kiss1-Cre* mice.

infertility induced by chronic hyperprolactinemia. The precise cellular mechanism by which prolactin suppresses kisspeptin neurons remains to be determined. Prolactin typically acts as a transcriptional regulator, with little or no evidence for rapid electrophysiological effect on kisspeptin neurons (22, 59), but prolactin may affect activity through changes in protein phosphorylation or gene transcription. Consistent with this idea, we have reported that prolactin induces pSTAT5 in most kisspeptin neurons in the rat arcuate, associated with a prolactin-induced reduction in kisspeptin immunoreactivity in this brain area (29).

The role of Prlr in RP3V kisspeptin neurons remains unclear. Prlr expressed in these neurons could not compensate for the absence of Prlr in the Arc kisspeptin neurons in terms of mediating a prolactin-induced suppression of pulsatile LH secretion. We have previously shown that elevated prolactin levels during lactation suppress kisspeptin expression in RP3V neurons, such that they no longer release kisspeptin even after direct stimulation (28). Based on the function of RP3V kisspeptin neurons to facilitate ovulation (60), this is likely to contribute (alongside a reduction of LH pulses) to the chronic suppression of reproduction by hyperprolactinemia in females. Physiologically, this is likely to be most relevant in establishing a period of anovulation during lactation, although there is uncertainty over the relative role of hyperprolactinemia or some other neurogenic effect of the suckling stimulus in the maintenance of lactational anovulation (19, 61, 62).

Our data establish that acute prolactin-induced suppression of pulsatile LH secretion is mediated by Prlr expressed on arcuate kisspeptin neurons, likely reducing the patterned afferent stimulation to GnRH neurons. This provides a mechanistic understanding of how inappropriately elevated prolactin causes infertility.

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