Hypothalamic Prolactin Regulation of Luteinizing Hormone Secretion in the Female Rat

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Prolactin (PRL) levels increase in response to long-term antipsychotic treatment that disrupts reproductive function. Recent evidence suggests that activation of central PRL receptors (PRLR) inhibits LH secretion and in ovariectomized rats. However, the mechanisms involved, the mode of LH secretion affected and relevance to hyperprolactinemia remain unknown. We therefore investigated the contribution of central PRL/PRLR signaling to the control of estradiol-induced surges of LH and PRL and pulsatile LH secretion under basal and hyperprolactinemic conditions. First, by subjecting ovariectomized estradiol-primed rats intracerebroventricularly administered with PRL to frequent blood sampling, we demonstrated that acute activation of hypothalamic PRLR disrupts pulsatile LH secretion. Pretreatment (intracerebroventricularly) with the pure PRLR antagonist, 1-9-G129R-hPRL, or the GABA, -aminobutyric acid receptor type A antagonist, bicuculline, blocked this effect. Next, we revealed that sustained blockade of hypothalamic PRLR using 1-9-G129R-hPRL augmented the magnitude of LH surges induced by estradiol benzoate and progesterone treatment and suppressed the concomitant surges of PRL. Finally, we determined that acute antagonism of central PRLR is insufficient to normalize the duration of the LH pulse interval prolonged as a result of hyperprolactinemia induced by chronic exposure to the atypical antipsychotic sulpiride. These data serve as the first evidence to suggest that PRL signaling through hypothalamic PRLR inhibits pulsatile secretion of LH in a GABA-aminobutyric acid receptor type A-dependent fashion and tonically restrains the magnitude of the LH surge. Furthermore, our results indicate that transient blockade of hypothalamic PRL/PRLR signaling is not an effective strategy for restoring LH pulsatility perturbed by chronic hyperprolactinemia. (Endocrinology 156: 0000–0000, 2015)

Pro prolactin (PRL) is a peptide hormone with diverse regulatory functions in the reproductive, nervous, and immune systems. It derives its name from its involvement in the control of lactation and mammary gland development (1). The anterior pituitary is the major origin of PRL and contains abundant lactotropes, specialized cells that secrete PRL into systemic circulation (2). The brain is a key target of PRL; circulating PRL is actively transported across the blood brain barrier into the cerebrospinal fluid (CSF) by PRL receptor (PRLR)-expressing cells of the choroid plexus (3). Furthermore, neural and glial cells within circumventricular nuclei have been shown to internalize and retain immunoreactive PRL from the CSF (4).

The plethora of biological effects exerted by PRL are initiated by the interaction between PRL and its receptor, PRLR, which exists in long and short isoforms in humans and rodents (5). The PRLR is an archetype of type 1 cytokine receptors and comprises homodimerized single-pass transmembrane chains of minimal complexity compared to other family members (6). Upon PRL binding, conformational changes occurring within the receptor-hormone complex impact ligand internalization and ultimately elicit biological responses through both receptor tyrosine kinase and non-tyrosine kinase pathways.

Abbreviations: aCSF, artificial CSF; ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus; CSF, cerebrospinal fluid; D2R, dopamine type 2 receptor; E2-17β-estradiol; E2B, E2 benzoate; GABA, -aminobutyric acid; icv, intracerebroventricular; KNDy, kisspeptin/neurokinin B/dynorphin A; LC, locus coeruleus; OVX, ovariectomized; P4, progesterone; PI3K, phosphatidylinositol-3-kinase; PRL, prolactin; PRLR, PRL receptor; pSTAT5, phosphorylated STAT5; SCN, suprachiasmatic nucleus; STAT5, signal transducer and activator of transcription-5; TIDA, tuberoinfundibular dopaminergic.
modulators trigger a combination of intracellular signaling cascades including Janus kinase-2/signal transducer and activator of transcription-5 (STAT5),Src kinase/phosphatidylinositol-3-kinase (PI3K)/Akt, MAPK and Nek3-vav2-Rac1 pathways (5). PRLRs are expressed throughout the hypothalamus in both long- and short-form isoforms (7–10), notably within neurons known to play important roles in the control of reproductive hormone secretion. Curiously, hypothalamic PRLRs have been shown to be twice as abundant in women than in men (11).

Although few GnRH neurons, major hypothalamic effectors of the hypothalamo-pituitary gonadal axis, express PRLRs (12), they receive ample projections from regulatory afferents that are PRLR positive: kisspeptin/neurokinin B/dynorphin A (KNDy) neurons of the arcuate nucleus (ARC), an essential component of the GnRH pulse generator; kisspeptin neurons of the anteromedial periventricular nucleus (AVPV), which are indispensable for mediating the positive-feedback effects of estradiol (E2) on GnRH secretion that results in the preovulatory LH surge; and γ-aminobutyric acid (GABA)ergic neurons of the AVPV that regulate GnRH neuron excitatibility (12).

Indeed, central (13) or peripheral (14) administration of ovine PRL prevents the occurrence of preovulatory LH surges in ovary-intact rats and down-regulates ARC kisspeptin expression and LH levels in ovariectomized (OVX) rats (15). Intracerebroventricular (ivc) administration of PRL also dose dependently induces the phosphorylation of STAT5 in the ARC and AVPV neurons of the OVX rat, and this effect is amplified by the replacement of E2 (16). A significant proportion of PRL-receptive ARC neurons are likely to be KNDy neurons because icv administration of PRL induced phosphorylated STAT5 expression in most kisspeptin-immunoreactive cells in this region (15).

Another group of highly PRL-receptive hypothalamic neurons are involved in the negative feedback of PRL on its own secretion. Tuberoinfundibular dopaminergic (TIDA) neurons project from the ARC to the median eminence, in which they secrete dopamine into the long portal vasculature (17). In addition, rodents possess tuberohypophysial and periventricular-hypophysial dopamine neurons (18), which innervate and secrete dopamine into short portal vessels within the neural and intermediate lobes of the pituitary that supply the anterior lobe (17). Dopamine released by these neuroendocrine systems activates dopamine type 2 receptors (D2R) on hypophysial lactotropes, tonically inhibiting PRL secretion (19). Curiously, KNDy neurons synapse with TIDA (20) and periventricular-hypophysial (21) neurons, and earlier studies have demonstrated projection of ARC dynorphinergic neurons to TIDA neurons (22). Moreover, recent evidence suggests that kisspeptin stimulates PRL release by inhibiting the activity of hypothalamic dopaminergic neurons in an E2-dependent fashion (23). Thus, the hypothalamic neuroendocrine systems regulating the secretion of gonadotropins and PRL appear to participate in regulatory cross talk that is subject to modulation by central PRL, albeit through yet unknown mechanisms.

LH surges occur after exposure to elevated E2 concentrations that stimulate de novo synthesis and release of progesterone (P4) by specialized neurons and astrocytes in the diencephalon (24–26). The positive feedback effects of E2 and P4 are then conveyed to GnRH neurons by kisspeptin neurons of the AVPV (27) subject to appropriate circadian disinhibition (28). Several lines of evidence implicate central PRL/PRLR signaling in the regulation of the LH surge: 1) hyperprolactinemia causes anovulation in women with microprolactinomas by inhibiting not only pulsatile LH secretion but also the midcycle LH surge (29, 30); 2) a high proportion of both ARC and AVPV kisspeptin neurons express PRLR in female rats (12); 3) lactating mice express 90% less kisspeptin mRNA and 60% less immunoreactive kisspeptin in AVPV neurons compared with diestrous controls (31, 32). However the role of central PRL in the regulation of gonadotropin surges remains obscure. Moreover, it has long been known that surges of PRL accompany preovulatory LH surges in rodents (33), ruminants (34), and humans (35) alike, and although the primary role of the PRL surge is apparently luteolytic (36), its potential effects at the hypothalamic level have not previously been considered.

To characterize the mechanism by which central PRL signaling regulates the GnRH pulse generator, we interrogated the hypothesis that GABAergic neurons mediate PRL-induced suppression of pulsatile LH secretion in OVX rats primed with low (dierstrus d 2) levels of E2 (OVX+E2 rats). Because the involvement of central PRL signaling in the regulation of gonadotropin surges is currently unknown, we then studied the effect of intracerebroventricularly administered, Δ1-9-G129R-hPRL, a unique pure PRLR antagonist, on the dynamics of LH surges induced by 17β-estradiol-3-benzoate (E2B) and P4 treatment (37) in OVX+E2 rats. Finally, we asked whether acute blockade of central PRLR signaling would reverse the inhibitory effect of chronic antipsychotic-induced hyperprolactinemia on the LH pulse so as to address the hypothesis that chronically elevated PRL acts centrally to suppress reproductive function.

Materials and Methods

Animals

Adult female Sprague Dawley rats (200–250 g) obtained from Harlan were housed under controlled conditions (12 h
light, 12 h darkness, with lights on at 7:00 AM; controlled ambient temperature 22°C ± 2°C and provided with standard laboratory chow (RM1; SDS Diets) and water ad libitum. Animals were group housed (maximum four per enclosure) prior to surgery and housed individually after surgery and during experimentation. All animal procedures were undertaken in accordance with the Animals (Scientific Procedures) Act, 1986 and were approved by the King’s College London Ethical Review Panel Committee.

Surgical procedures

All surgical procedures were carried out under general anesthesia induced by ketamine (100 mg/kg ip; Pharmacia and Upjohn) coadministered with xylazine (Rompun; 10 mg/kg ip; Bayer); supplementary injections of ketamine (10 mg, ip) were administered to maintain anesthesia as required. Each rat was injected sc with 0.4 mL/kg Duxhamox LA antibiotic suspension (200 mg/mL procaine benzylpenicillin, 250 mg/mL dihydrostreptomycin-sulfate; Fort Dodge Animal Health) prior to surgery. After each surgical procedure, animals were allowed to recover from anesthesia on a heated pad until fully conscious. Postoperative analgesia was provided by means of sc administration of carprofen (Rimady; 4.4 mg/kg; Pfizer Animal Health) daily for 3 days.

Two weeks before experiments took place, the rats were bilaterally OVX and implanted with subcutaneous E$_2$-filled SILASTIC brand capsules (Dow Corning), which produce circulating concentrations of E$_2$ within the range observed during the diestrous (d 2) phase of the estrous cycle (35.8 ± 1.2 pg/mL), as previously described (38). Rats were then implanted with unilateral guide cannulae (22 gauge; Plastics One), stereotaxically targeted toward the left lateral cerebral ventricle for microinfusion of pharmacological agents. The stereotaxic coordinates for cannula placement was confirmed by the observation of gravitational meniscus movement upon insertion of an internal injection cannula (Plastics One) with extension tubing preloaded with artificial CSF (aCSF). A 20-mm stainless steel slotted screw (Insect Laboratories) was affixed to the surface of the skull posterior to the guide cannula and both were secured using dental cement (Simplex Rapid; Dental Filling). The icv guide cannulae were then fitted with obturators (Plastics One) to maintain patency. After a 10-day recovery period, the rats were implanted with two custom-made cardiac catheters via the internal jugular veins to enable simultaneous automated serial blood sampling for determination of LH levels and manual withdrawal of blood for determination of PRL levels (40). The catheters were exteriorized at the back of the head and enclosed within a 30-cm-long light-weight metal spring tether (Insect Laboratories) secured to the slotted screw cranial attachment with surgical ligature. The distal end of the tether was attached to a dummy swivel (Insect Laboratories), allowing the rat to move freely. After surgery, animals were housed singly. Experimentation commenced after a further 3 days of recovery from surgery.

Experimental design

On the morning of experimentation, an icv injection cannula with extension tubing, preloaded with a combination of drug solutions, was inserted into the guide cannula. The distal end of the tubing was filled with aCSF. The remainder of the tubing was filled with sterile water, with 5 μL air separating the water and aCSF, which allowed the progress of injections to be monitored. The tubing was extended outside the cage and connected via one channel of a two-channel fluid swivel (Insect Laboratories) to a 25-μL syringe (Hamilton) prefilled with sterile water to allow remote microinfusion without disturbing the rats during the experiment. One of the two cardiac catheters was then attached via the second channel of the fluid swivel to a computer-controlled automated blood sampling system, which enables the intermittent withdrawal of small blood samples (25 μL) every 5 minutes for 4–6 hours without disturbing the rats. After removal of each 25-μL blood sample, an equal volume of heparinized saline (50 U/mL normal saline; CP Pharmaceuticals) was automatically infused to maintain patency of the catheter and blood volume. Once connected, the animals were left undisturbed for 1 hour before blood sampling was initiated (between 9:00 and 10:00 AM). Drug treatments were administered after a period of control blood sampling manually over 5 minutes. Additional blood (50 μL) was sampled manually via the second cardiac catheter at specified time points, and each withdrawal was followed by the administration (iv) of 50 μL heparinized saline. Plasma obtained by centrifugation of manually withdrawn blood samples was frozen at −20°C and later assayed to determine concentrations of PRL by means of a RIA. Automatically sampled blood was frozen at −20°C and later assayed to determine LH concentrations by means of RIA. To minimize animal suffering in line with the Animals (Scientific Procedures) Act, 1986, rats were reused as part of different treatment groups within experiments, but not between experiments, and were allowed to recover for at least 3 days between bleeding procedures.

Drugs and hormones

Δ1-9-G129R-hPRL, recombinant human PRL with Gly$^{129}$ replaced with Arg and nine N-terminal residues deleted, is a pure PRLR antagonist (41). Δ1-9-G129R-hPRL was expressed in Escherichia coli as inclusion bodies, using the pT7L expression vector, as described previously (42), and purified using an ion exchange column (Hi Trap Q-Sepharose; GE Healthcare Life Sciences). Lyophilized aliquots of Δ1-9-G129R-hPRL were reconstituted with sterile water and diluted with aCSF. Recombinant rat PRL (ABC2856; Source BioScience Life Sciences) was reconstituted from lyophilized stocks with sterile water and diluted with aCSF. The selective GABA$_{A}$R antagonist bicuculline (1(S),9(R)-(−)-bicuculline methobromide; Sigma-Aldrich) was dissolved in sterile water and diluted with aCSF. The D$_3$R antagonist sulpiride ([S]-(−)-sulpiride; sc-258111; Santa Cruz Biotechnology) was solubilized with 1 M tartaric acid (L-(+)-tartaric acid (sc-218622; Santa Cruz Biotechnology) and diluted with saline, the final concentration of tartaric acid being 10 mM.

Effect of central administration of PRL on pulsatile LH secretion in OVX+E$_2$ rats

A total of 7 OVX+E$_2$ rats were used in this experiment; a crossover design ensured that each animal was randomly assigned to a treatment group and subsequently to its counterpart control group. The treatment groups were as follows: Δ1-9-G129R-hPRL (antag) + aCSF (n = 4), bicuculline (bic) + aCSF (n = 3), aCSF + PRL (n = 5), antag + PRL (n = 7), and bic + PRL (n = 5). All treatments were administered icv in a volume of
4 μL aCSF. One hour 40 minutes into a 6-hour blood sampling procedure, rats were pretreated with 20 μg Δ1-9-G129R-hPRL, 500 ng bicuculline, or 4 μL aCSF. Twenty minutes later, the animals received a further icv injection of 5 μg PRL or 4 μL aCSF. Additional blood samples for measurement of PRL levels were withdrawn at 60, 135, 150, 180, 240, and 360 minutes relative to the time when the automated blood sampling was initiated.

**Effect of hypothalamic PRLR antagonism on LH and PRL surges induced by E₂B and P₄ treatment in OVX+E₂ rats**

To induce concomitant LH and PRL surges, OVX+E₂ rats were subjected to gonadal steroid priming according to a protocol described previously (43). A total of 10 animals were injected (sc) with 2 μg E₂B (Sigma-Aldrich) in 100 μL arachis oil (Sigma-Aldrich) at approximately 9:00 AM daily for 2 days. At 9:00 AM on the morning of the experiment, the rats received 500 μg P₄ (Sigma-Aldrich) in 100 μL arachis oil (Sigma-Aldrich), sc. One hour 30 minutes later, a 1-hour control blood sampling procedure was initiated. Three hours after the P₄ administration, the rats were injected (icv) with 20 μg Δ1-9-G129R-hPRL in 4 μL aCSF (n = 7) or 4 μL aCSF (n = 3). One hour later (240 min after P₄ administration), a further 6 hours of serial blood sampling was initiated. At the same time, a 3-hour infusion of 60 μg Δ1-9-G129R-hPRL in 12 μL aCSF (n = 7) or 12 μL aCSF (n = 3), driven by a syringe pump (PHD 2000; Harvard Apparatus), was started. Additional blood samples for measurement of PRL levels were withdrawn at 60, 120, 240, 300, 360, 420, 480, 540, and 600 minutes relative to the time of P₄ administration.

**Effect of hypothalamic PRLR antagonism on pulsatile LH secretion in chronically hyperprolactinemic OVX+E₂ rats**

A total of 15 animals were used in this experiment. To render OVX+E₂ rats chronically hyperprolactinemic, sc injections of sulpiride (5 mg/kg in 450 μL 10 mM tartaric acid) were given at 9:00 AM and 5:00 PM hours daily for at least 11 days prior to experimentation as well as on the day of the experiment (n = 8). This protocol has been previously demonstrated to induce chronic hyperprolactinemia in OVX+E₂ rats (16). Control rats (n = 7) received 450 μL 10 mM tartaric acid (sc) at 9:00 am and 5:00 PM daily for at least 11 days prior to experimentation as well as on the day of the experiment. Rats were subjected to a 4-hour serial blood sampling procedure that commenced at 10:00 AM. The animals received four consecutive icv injections of 20 μg Δ1-9-G129R-hPRL in 4 μL aCSF or 4 μL aCSF at 20-minute intervals between 12:00 and 3:00 PM. Additional blood samples for measurement of PRL levels were withdrawn hourly between 9:30 AM and 5:30 PM. The experiment followed a crossover design whereby all animals received Δ1-9-G129R-hPRL and six of the eight sulpiride-treated rats and five of the seven control rats were randomly selected to receive aCSF during separate trials at least 3 days apart.

**Hormone RIA**

Double-antibody RIAs supplied by the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, California) were used to determine LH and PRL concentrations in the 25-μL whole-blood samples and 10-μL plasma samples, respectively. Values are expressed in terms of the standard preparations NIDDK-rPRL-RP-3 and NIDDK-rLH-RP-3. The limit of sensitivity of the LH RIA was 0.093 ng/mL and the inter- and intraassay variations were 6.8% and 8.0%, respectively. The limit of sensitivity of the PRL RIA was 4.9 ng/mL. The intraassay variation was 8.9% and all PRL values were obtained from a single assay.

**Data analysis**

Detection of LH pulses was facilitated by the use of the algorithm ULTRA (44). Two intraassay coefficients of variation (2 × c_v) of rat plasma pools, with LH concentrations falling within the log phase of the LH RIA standard curve were used as the reference threshold for pulse detection. The effect of treatments on LH pulse frequency was analyzed by comparing the mean LH pulse interval in the 2-hour period preceding treatment with that in 2-hour posttreatment period(s). The period duration (in minutes) was divided by the number of LH pulses detected in each of these periods to give the appropriate LH pulse interval. When there were no LH pulses evident during the first 2-hour posttreatment period, the LH pulse interval assigned to this period was taken as the interval from the onset of treatment to the first LH pulse in the second 2-hour posttreatment period. The significance of the effect of treatments on LH pulse frequency was compared with the respective negative control group at the same time points as well as with the mean pulse interval during the 2-hour pretreatment period in the same treatment group. Statistical significance was tested using a one-way ANOVA with Bonferroni post hoc test. P < .05 was considered statistically significant. Values given in the text and figures represent mean (±SEM).

PRL levels in all experiments, as well as surge-like LH levels, were compared using a two-way ANOVA with repeated measures (influence of time and treatment) with Holm-Sidak pairwise multiple comparison. Overall significance threshold was P < .05. In addition, cumulative changes in PRL and LH levels were expressed as area under the curve for individual treatment groups, which were compared using a paired, two-tailed Student’s t test (two groups) or a one-way ANOVA with Bonferroni post hoc test (more than two groups). P < .05 was considered statistically significant. Values given in the text and figures represent mean (±SEM).

**Results**

**Effect of central administration of PRL on pulsatile LH secretion in OVX+E₂ rats**

No effect of icv Δ1-9-G129R-hPRL pretreatment on the LH pulse was observed in rats administered with aCSF (representative example in Figure 1A), at least during the first 4 hours 20 minutes after the injection. Bicuculline also had no significant effect on pulsatile LH secretion (representative example in Figure 1B). Administration (icv) of PRL suppressed pulsatile LH secretion for a period of 1–2 hours (representative examples in Figure 1, C and D). No LH pulses were detectable during the period of PRL-induced suppression, the onset of which was immediate.
upon administration in three of the five animals (representative example in Figure 1C) and delayed by 10–60 minutes in two of the five animals (representative example in Figure 1D). A gradual recovery of the LH pulse was seen in the second 2 hours after the injection in all five animals. Pretreatment with either Δ1-9-G129R-hPRL (representative example in Figure 1E), or bicuculline (representative example in Figure 1F) completely blocked the PRL-induced suppression of the LH pulse. The results of this experiment are summarized in the histogram (Figure 1G). None of the treatments had a significant effect on circulating PRL levels, which fluctuated between 20 and 100 ng/mL in all treatment groups (Figure 1H).

Effect of hypothalamic PRLR antagonism on LH and PRL surges induced by E$_2$B and P$_4$ treatment in OVX+E$_2$ rats

Treatment of OVX+E$_2$ rats with E$_2$B and P$_4$ induced afternoon surges of LH (Figure 2A) and PRL (Figure 2B). LH levels increased steadily between 300 and 480 minutes after P$_4$ (2:00 PM and 5:00 PM, respectively) in all rats, plateauing at approximately 15 ng/mL in rats treated with aCSF and 27 ng/mL in Δ1-9-G129R-hPRL-treated rats and subsequently declining. The magnitude of the LH surge was significantly greater in rats treated with Δ1-9-G129R-hPRL than in aCSF-treated controls (Figure 2A). Treatment with Δ1-9-G129R-hPRL did not alter the time of LH surge onset. PRL levels in aCSF-treated controls rose significantly above baseline at 420 minutes after P$_4$ (4:00 PM), remaining elevated at 480 minutes (5:00 PM) and declining thereafter (Figure 2B). In rats administered with Δ1-9-G129R-hPRL, PRL levels remained at baseline throughout the blood sampling procedure (Figure 2B).

Effect of hypothalamic PRLR antagonism on pulsatile LH secretion in chronically hyperprolactinemic OVX+E$_2$ rats

Chronic sulpiride-induced hyperprolactinemia perturbed the regularity of LH pulses and increased the duration of the interval between them from approximately 28 minutes to more than 45 minutes (Figure 3, A–E). Acute treatment with Δ1-9-G129R-hPRL had no detectable effect on LH pulse frequency, neither in rats administered with vehicle nor in those treated with sulpiride (Figure 3, A–E). PRL levels in chronic sulpiride animals (200–1000 ng/mL) were significantly higher than those in chronic

Figure 1. Effect of central administration of PRL on pulsatile LH secretion in OVX+E$_2$ rats. Representative LH profiles demonstrating the effect of icv administration (long arrows) of vehicle (aCSF; A and B) or PRL (C–F), after icv pretreatment (short arrows) with one of the following: 1) the pure PRLR antagonist, Δ1-9-G129R-hPRL (antag; A and E); 2) the selective GABA$_A$R antagonist, bicuculline (bic; B and F); or 3) vehicle (aCSF; C and D) on pulsatile LH secretion in OVX+E$_2$ rats. The suppression of the LH pulse by PRL was blocked by both the PRLR and the GABA$_A$R antagonists, as summarized in panel G. None of the


Figure 1 (Continued). treatments significantly affected PRL secretion (H). The histogram (G) depicts group means (±SEM). White numbers within the black bars (G) denote the size of the treatment groups. *, P < .05 vs 2-hour baseline control period within the same treatment group as well as vs the same 2-hour period within all other treatment groups.
vehicle controls (<200 ng/mL) throughout the blood-sampling procedure (Figure 3, F and G). In sulpiride-treated animals, PRL levels were highest at 9:30 AM (30 min after the morning sulpiride injection), declining through the afternoon and increasing again at 5:30 PM (30 min after the evening sulpiride injection). PRL levels in vehicle-treated controls did not change significantly through the blood-sampling procedure, although a non-significant trend whereby PRL levels were higher at 9:30 AM than at subsequent time points was observed. Again, acute administration of Δ1-9-G129R-hPRL had no effect on PRL levels in both vehicle- and sulpiride-treated rats (Figure 3, F and G).

Discussion

In an attempt to broaden the understanding of the roles of central PRL/PRLR signaling in regulating the reproductive system, we are the first to report that icv administration of OVX+E2 rats with exogenous PRL suppresses pulsatile LH secretion in a GABAergic-dependent fashion. We have also demonstrated that central PRLR-induced suppression of the LH pulse is fully blocked by the antagonism of central PRLR. Taken together, our findings suggest that endogenous hypothalamic GABA release is rapidly triggered in response to exogenous PRL activation of central PRLR and that signaling through GABA receptors is essential for the LH pulse inhibition induced by central PRLR administration. However, the PRL-receptive GABAergic neurons and their efferent targets that modulate LH secretion remain to be identified. An equal dose of PRL to that used in experiments we describe here has previously been demonstrated to potentiate cFos (45) and phosphorylated STAT5 (pSTAT5) (46) immunoreactivity in several hypothalamic nuclei known to contain GABAergic neurons, including the ARC and AVPV. Of these, the ARC population was identified to express tyrosine hydroxylase (an enzyme involved in the catalysis of the rate limiting stage of catecholamine synthesis) (45), suggesting a dopaminergic phenotype. However, a wealth of evidence indicates that some ARC tyrosine hydroxylase neurons, including TIDA neurons, are also GABAergic (47–51).

Arcuate GABA signaling is known to negatively regulate pulsatile LH secretion: microimplantation of crystalline GABA in the ARC of OVX rats suppresses LH pulse frequency and mean LH levels (52). This effect is mediated by GABA receptors because administration of the selective GABA receptor agonist, muscimol, suppresses both the electrophysiological (multiunit activity volleys in the mediobasal hypothalamus) and endocrine (pulsatile LH secretion) correlates of the GnRH pulse generator in OVX rats (53). More recently GABA has been shown to inhibit the spontaneous firing activity of murine KNDy neurons (54) and central agonism of GABA receptors, but not GABA receptors, attenuated kisspeptin-induced gonadotropin secretion in male rats (55). Central PRL administration has recently been shown to suppress ARC kisspeptin expression and mean LH levels in OVX rats (15). These findings are in agreement with the inhibition of kisspeptin mRNA and immunoreactivity in the ARC, as well as the suppression of
pulsatile LH secretion, reported throughout lactation in suckled OVX rats (56, 57), which is well known to markedly elevate circulating PRL (58) and CSF GABA levels. However, no differences in ARC GABA turnover were detected between diestrous and lactating rats, despite elevated PRL and suppressed LH secretion in the latter (60), which suggests that PRL-induced activation of GABAergic signaling might occur elsewhere. GABAergic neurons of the AVPV, unlike those in surrounding nuclei, are highly PRL receptive (12) and are known to innervate GnRH perikarya (61). Both GABA_A R (62) and GABA_B R (63, 64) have been shown to be expressed by GnRH neurons. However, it has been reported that the GABAergic signal supplied to GnRH neurons by the AVPV is predominantly stimulatory (61). These conundrums necessitate investigation into the anatomy and function of PRL-responsive GABAergic neurons we implicate in the suppression of pulsatile LH secretion induced by acute central administration of PRL.

We did not detect changes in endogenous PRL secretion in response to acute central administration of exogenous PRL. Neither did the acute Δ1-9-G129R-hPRL blockade of central PRLR influence PRL secretion. This is perplexing in light of extensive evidence for the short-loop negative feedback effect of PRL on its own secretion (65–68). However, classic studies by Moore and colleagues (69–71) evidenced consistently delayed feedback effects of central or peripheral PRL administration in rats, as revealed
by median eminence dopamine synthesis and turnover increasing significantly as long as 12–24 hours after treatment. Moore and colleagues (72, 73) subsequently revealed a more rapid activation of PRL feedback in animals with basal PRL secretion suppressed by bromocriptine (D2R agonist) treatment or hypophysectomy, although even that was detectable only after 4 hours. Thus, the absence of a short-loop negative feedback effect of central PRLR activation, or that of their blockade, on circulating PRL levels in normoprolactinemic OVX+E2 rats within the examined time frame is expected.

Having established a tentative mechanism for PRL-induced suppression of pulsatile LH secretion and confirmed the pure antagonistic properties of Δ1-9-G129R-hPRL in our experimental setting, we interrogated the hypothesis that PRL found endogenously within the central nervous system modulates LH and PRL surges. There have been several prior attempts to investigate the involvement of central PRL signaling in the regulation of the LH surge (16, 74, 75). However, these studies involved the augmentation of PRL signaling beyond that found under physiologic conditions. Here we demonstrate that blockade of central PRLR augments the magnitude of the LH surges induced by E2B and P4 treatment, without altering the time of its onset, and blocks the concomitant PRL surges induced by E2B and P4 treatment, without altering the examined time frame is expected.

Another potential mechanism that might underlie the Δ1-9-G129R-hPRL-induced amplification of the LH surge involves changes in the sensitivity of noradrenergic neurons of the locus coeruleus (LC) to P4. These neurons down-regulate their expression of P4 receptor on proestrus afternoon (88), concomitant with the PRL surge and increased cFos expression in the LC (89), and are known to provide stimulatory noradrenergic input to GnRH neurons during the LH surge (90, 91). Because no data are available on the PRL sensitivity of the noradrenergic LC, it is unclear at what level PRL or Δ1-9-G129R-hPRL would be able to modulate these pathways. However, recent evidence again implicates the highly PRL-receptive nature of hypothalamic GABA neurons (12), it is logical to speculate that PRL regulation of de novo progesterone synthesis by neurons and glia could be mediated by GABAergic afferents (87).

Although pretreatment with Δ1-9-G129R-hPRL augmented the magnitude of the LH surge, it had no effect on its time of onset. Similar observations were reported in GnRH-primed, progesterin F2α-treated lactating yaks, in which suppression of PRL secretion with quinagolide (another D2R agonist) augmented the magnitude of LH surges induced by GnRH administration but did not affect its onset (93). The onset of the preovulatory LH surge in nocturnal rodents is timed to coincide with the reduction in daylight by the circadian pacemaker within the suprachiasmatic nucleus (SCN) the hypothalamus (94). Arginine vasopressin neurons of the SCN markedly increase the sensitivity of GnRH neurons to stimulation by AVPV kisspeptin neurons and disinhibit GnRH secretion from tonic suppression by RF-amide related peptide 3 neurons in a time-gated fashion (95). We expect the association...
between disruption of central PRL/PRLR signaling and augmentation of the magnitude of the LH surge without a change in its timing, which we and others (93) have demonstrated, to reflect the suppressive effect of a centrally penetrating PRL tone on the ability of AVPV kisspeptin neurons to elicit GnRH release during a time window governed by neurons of the SCN, without affecting the time-restricting function of the SCN neurons per se. Indeed, AVPV kisspeptin neurons (12), but not SCN neurons (96), have been shown to express PRLR, confirming that the circadian pacemaker is not subject to regulation by central prolactin.

The absence of PRL surges in Δ1-9-G129R-hPRL-treated animals is perhaps best explained by the inability of centrally transported PRL to activate the positive oxytocin-PRL feedback loop recently suggested by Kennett and McKee (97). In our experiments, both control and Δ1-9-G129R-hPRL-treated rats were exposed to P₄ levels capable of suppressing TIDA neuron dopamine output and thereby permissive to PRL surge generation (98–101). However, blockade of central PRLR presumably extended to those expressed by hypothalamic oxytocin neurons (102) that secrete oxytocin in response to elevated E₂ (103, 104) and PRL (97, 105) levels. Oxytocin is a potent PRL secretagogue (106, 107), its stimulatory effect on PRL secretion is augmented during proestrus (108), and oxytocin antagonists block PRL surges (109–111). Thus, Δ1-9-G129R-hPRL-treated, but not control rats, were probably deprived of oxytocinergic stimulation of surge release of PRL. An alternative hypothesis implicates GABA/GABA₄R signaling: central administration of bicuculline has been shown to block afternoon surges of PRL by suppressing TIDA neuron dopamine output and thereby permissive to PRL surge generation (98–101). However, blockade of central PRLR presumably extended to those expressed by hypothalamic oxytocin neurons (102) that secrete oxytocin in response to elevated E₂ (103, 104) and PRL (97, 105) levels. Oxytocin is a potent PRL secretagogue (106, 107), its stimulatory effect on PRL secretion is augmented during proestrus (108), and oxytocin antagonists block PRL surges (109–111). Thus, Δ1-9-G129R-hPRL-treated, but not control rats, were probably deprived of oxytocinergic stimulation of surge release of PRL. An alternative hypothesis implicates GABA/GABA₄R signaling: central administration of bicuculline has been shown to block afternoon surges of PRL by suppressing the activity of TIDA neurons (112) and to augment LH surge magnitude without advancing its onset (113) in rats. Furthermore, we have shown that icv pretreatment with either Δ1-9-G129R-hPRL or bicuculline restores LH pulsatility suppressed by acute central PRLR administration, confirming the close functional relationship between hypothalamic PRL/PRLR and GABA/GABA₄R signaling implied by the PRL-receptive anatomy of GABAergic afferents to GnRH neurons (12).

These findings are consistent with a role for endogenous PRL in attenuating the extent of GnRH neurosecretion underlying surges of LH by directly stimulating hypothalamic release of GABA and highlight the importance of central PRL/PRLR signaling in stimulating its own secretion through positive-feedback effects on hypothalamic GABA neurons (that suppress TIDA inhibition of PRL secretion) and/or oxytocin neurons (which stimulate pituitary PRL release). Future studies should confirm whether the mechanism of Δ1-9-G129R-hPRL-induced blockade of the PRL surge involves the disruption of positive oxytocin-PRL feedback and/or that of GABAergic disinhibition of PRL secretion from the dopamine tone.

Hyperprolactinemia, including that induced by antipsychotic treatment, suppresses reproductive function and, paradoxically, the only effective means of treatment are dopamine receptor agonists, which exacerbate psychotic symptoms and are therefore contraindicated (114–116). Having demonstrated that central administration of PRL inhibits the pulsatile secretion of LH in normoprolactinemic OVX+E₂ rats, we reasoned that central PRLR might resemble a useful therapeutic target for the management of reproductive defects caused by antipsychotic-induced hyperprolactinemia. To test this hypothesis, we administered OVX+E₂ rats receiving long-term twice-daily sulpiride treatment with Δ1-9-G129R-hPRL into the cerebroventricular system. We confirmed previous observations (16) that chronic administration of sulpiride is an effective means of inducing hyperprolactinemia and reducing the frequency of LH pulses in OVX+E₂ rats. However, four consecutive injections of 20 µg Δ1-9-G129R-hPRL given every 20 minutes neither altered the PRL levels elevated by chronic exposure to sulpiride nor reversed the inhibitory effect of hyperprolactinemia on LH pulse frequency. These data demonstrate that acute blockade of central PRLR is not sufficient to reverse the effect of long-term hyperprolactinemia on pulsatile LH secretion.

Relative to the long-term changes in the chemistry of ARC neurons induced by chronic hyperprolactinemia (117), the limited duration of PRLR antagonism provided by acute icv administration of Δ1-9-G129R-hPRL might be sufficient to transiently disinhibit ARC kisspeptin expression from tonic PRL-induced suppression (15). However, if that were the case, such changes would be detectable only several hours after treatment and would not necessarily restore the LH pulse to frequencies observed in normoprolactinemic controls. In the context of the wider literature, these data suggest that the mechanism by which chronic hyperprolactinemia perturbs reproductive function involves long-term genomic changes in the cross talk between populations of PRL-sensitive hypothalamic neurons, which are not reversible by the acute blockade of central PRLR. To further address the contribution of central PRLR to the suppressive effects of hyperprolactinemia on reproductive function, selective inducible knockdown of PRLR within discrete hypothalamic nuclei in chronically hyperprolactinemic rats is advised.

In summary, we have shown that the activation of central PRLR leads to the acute suppression of LH pulsatility and demonstrated that GABA/GABA₄R signaling is an indispensable part of the mechanism involved. These data raise the possibility that PRL-receptive GABAergic neurons may mediate inhibition of fertility attributed to ele-
vated PRL. Moreover, we provide the first evidence that central PRL signaling provides tonic inhibition of the LH surge and is necessary for the generation of the PRL surge that tends to coincide with it. Finally, we report a lack of effect of acute blockade of central PRLR on pulsatile LH secretion and PRL levels in hyperprolactinemic rats. These observations assist the interpretation of and provide a context to previous reports on the role of PRL in modulating hypothalamic circuits important in regulating mammalian reproduction.

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