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Prolactin Regulation of Gonadotropin-Releasing Hormone Neurons to Suppress Luteinizing Hormone Secretion in Mice

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Hyperprolactinemia causes infertility, but the mechanisms involved are not known. The present study aimed to determine whether and how prolactin may influence LH secretion in the adult female mouse. Using ovariectomized, estrogen-treated (OVX+E) mice, we found that 7 d of intracerebroventricular prolactin potently suppressed serum LH levels ($P < 0.05$). To examine whether this central action of prolactin may involve the GnRH neurons, the effects of acute and chronic prolactin on cAMP response element-binding protein phosphorylation (pCREB) in GnRH neurons were examined using dual-label immunocytochemistry. In diestrous and OVX+E mice, a single sc injection of ovine prolactin resulted in a significant ($P < 0.05$) doubling of the number of GnRH neurons expressing pCREB. OVX+E mice treated with five injections of ovine prolactin over 48 h showed a 4-fold increase in the number of GnRH neurons with pCREB. To determine whether GnRH neurons might be regulated directly by prolactin, we examined prolactin receptor (PRL-R) mRNA expression in green fluorescent protein-tagged GnRH neurons by single-cell RT-PCR. As a positive control, PRL-R mRNA was measured in arcuate dopaminergic neurons obtained from green fluorescent protein-tagged tyrosine hydroxylase neurons. Three of 23 GnRH neurons (13%) were identified to express PRL-R transcripts, whereas nine of 11 arcuate dopaminergic neurons (82%) were found to coexpress PRL-R mRNA. These data demonstrate that prolactin suppresses LH levels in the mouse, as it does in other species, and indicate that it acts centrally to regulate intracellular signaling within GnRH neurons. This is likely to occur, at least in part, through the direct regulation of a subpopulation of GnRH neurons. (*Endocrinology* 148: 4344–4351, 2007)

HYPERPROLACTINAEMIA IS A WELL-established cause of infertility in both male and female mammals. Elevated prolactin may impact reproduction through an action on the GnRH neurons of the hypothalamus and/or on the pituitary gland to affect secretion of the gonadotrophins, LH, and FSH (1, 2). In humans, hyperprolactinemia is associated with a marked reduction in both the frequency and amplitude of LH pulses (3, 4), indirectly suggesting that both the brain and pituitary might be targets for prolactin. Importantly, pulsatile GnRH replacement can reverse the infertility induced by hyperprolactinemia (4–6), suggesting that prolactin-induced suppression of GnRH release is the proximal cause of infertility. The suppressive effects of prolactin upon reproduction have also been observed in animal models. Prolactin suppresses both the frequency and amplitude of LH pulses in male and female rats (7–10) and direct measurements of GnRH secretion into the portal blood have revealed a prolactin-induced suppression of GnRH release (11–14). Furthermore, hyperprolactinemia has been shown to prevent the castration-induced increase in GnRH mRNA expression in rats (15).

The mechanisms(s) by which prolactin inhibits GnRH neurons remain poorly understood. Whereas we have observed prolactin receptor (PRL-R) expression in the rat hypothalamus (16, 17), expression in GnRH neurons has not been demonstrated. PRL-R are expressed in GT1 cells, an immortalized cell line derived from mouse GnRH neurons, and prolactin potently suppresses GnRH biosynthesis and secretion in these cells (18). Although GT1 cells are quite different from adult GnRH neurons (19), these data suggest the possibility that prolactin may directly regulate GnRH neurons *in vivo*. Prolactin has been observed to influence γ-aminobutyric acid (20), opioid (21), neuropeptide Y (22), and dopaminergic (23) neuronal systems, all of which are implicated in the regulation of GnRH neurons under certain conditions. Hence, it seems likely that prolactin could also act to influence GnRH neurons indirectly through these afferent pathways.

Much of the difficulty in understanding the functioning of the GnRH neuronal network has come from an inability to undertake detailed investigations on native GnRH neurons *in situ*. The recent application of transgenic technologies to this problem has opened the door to the molecular and cellular investigation of the GnRH neuronal phenotype in the mouse (24, 25). There has been some controversy in the field, however, as to whether prolactin actually suppresses fertility in mice, as it does in other species. Some studies have failed to observe a prolactin-induced suppression of LH secretion

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Abbreviations: CREB, cAMP response element-binding protein; dNTP, deoxynucleotide triphosphate; GFP, green fluorescent protein; icv, intracerebroventricular; oPRL, ovine prolactin; OVX+E, ovariectomized, estrogen treated; pCREB, phosphorylated CREB; PRL-R, prolactin receptor; rPOA, rostral preoptic area; RT, reverse transcriptase; STAT, signal transducer and activator of transcription; TH, tyrosine hydroxylase.

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in mice (26), whereas others have demonstrated a clear suppression of fertility due to elevated prolactin (27, 28). The aim of the present study was to evaluate this issue in female C57BL/6J mice, with the goal of establishing a mouse model that would allow further investigation of prolactin action on GnRH neurons in transgenic mice. We report here that prolactin does indeed suppress LH secretion in the mouse and provide evidence that this occurs at least in part through a direct action on a subpopulation of GnRH neurons.

Materials and Methods

Animals

Adult female C57BL/6J mice (age 6–8 wk, weighing 24–28 g), were group housed under conditions of controlled temperature (21 ± 1 C) and lighting (12-h light, 12-h dark cycle). Stages of the estrous cycle were monitored by daily vaginal smears, and where indicated, animals were used in the diestrous phase of the estrous cycle. Groups of animals were ovariectomized and treated with a subcutaneous implant containing 17β-estradiol [silicon tubing, inner diameter 1.0 mm; outer diameter 2.125 mm; 12.5 mm long, containing 50 μg/ml mixed in silicon adhesive (29)] to maintain low physiological levels of plasma estradiol (OVX+E). Animals were used 5–7 d after ovariectomy.

Experiment 1: effect of intracerebroventricular (icv) prolactin infusion on LH secretion in ovariectomized mice

To determine whether chronic prolactin infusion into the brain could suppress LH secretion in mice, OVX+E mice were anesthetized and icv cannulae were implanted into the left lateral ventricle using stereotaxic coordinates (1.3 mm lateral to Bregma, 5 mm below skull) (30). Cannulae were attached to a sc implanted Alzet minipump (model 1002, 0.25 μl/h) containing ovine prolactin (oPRL), such that animals were treated with 625 ng/h for 7 d. Animals were then killed by decapitation and trunk blood collected and allowed to clot at 4 C. Serum LH levels were measured in 40-μl sample volumes by RIA. Iodinated rat LH (NIDDK-rat LH-I-10) was used as tracer and primary antisera was National Institute of Diabetes and Digestive and Kidney Diseases rabbit antirat LH-S11 (final dilution 1:400,000). Values are expressed in terms of mouse LH Reference Preparation, provided by A. F. Parlow (National Hormone and Peptide Program, Torrance, CA). All samples were measured in a single assay, with an intraassay coefficient of variation of 12%.

Experiment 2: prolactin-induced phosphorylation of cAMP response element-binding protein (CREB), in vivo

The phosphorylation of CREB can be used as a broad spectrum indicator of altered signaling events within cells (31) including GnRH neurons (32). As such, we monitored the CREB phosphorylation status of GnRH neurons to examine whether changes in circulating prolactin influences signaling in these cells. Groups of diestrous or OVX+E mice were injected sc with 50 μg oPRL (or saline vehicle as a control) and 60 min later perfused transcardially with 4% paraformaldehyde. To examine more chronic actions of prolactin, three more groups of OVX+E animals were treated twice daily for 48 h with either oPRL (50 μg/injection, sc), bromocriptine (100 μg/injection sc), or saline-vehicle controls. Bromocriptine is a dopamine D2 receptor agonist, known to inhibit endogenous prolactin secretion from the pituitary gland. It was used to ensure that multiple handlings for injections did not result in stress-induced increases in endogenous prolactin levels and hence potentially affect phosphorylated CREB (pCREB) expression in controls. The final injections were administered 1 h before perfusion, as described above. Brains were postfixed in the same fixative for 2 h and then cryoprotected in 30% sucrose overnight. Four consecutive sets of 30-μm coronal sections through the preoptic area and rostral hypothalamus were cut using a sliding microtome. One set of sections was used to evaluate CREB expression in GnRH neurons and another to examine pCREB expression.

Free-floating, dual-labeling, peroxidase immunocytochemistry was undertaken in the same manner as reported previously (32). In brief, after a 0.1% H_2O_2/40% methanol/Tris-buffered saline wash, all sections from one set of sections were incubated in one of the primary antibodies (pCREB, 1:100 or CREB, 1:100; Cell Signaling Technology, Inc., Beverly, MA) for 48 h at 4 C. This was followed by biotinylated goat antirabbit IgGs (1:200 for 2 h; Vector Labs, Peterborough, UK) and the Vector Elite avidin-biotin-horseradish peroxidase complex (1:200 for 2 h). Peroxidase labeling was then visualized with nickel-diaminobenzidine tetrahydrochloride using glucose oxidase. Sections were then treated with 0.1% H_2O_2/40% methanol/Tris-buffered saline to quench any remaining peroxidase activity and processed further for GnRH immunoreactivity with the LR1 antibody (1:20,000) followed by peroxidase-labeled antirabbit IgGs and revealed using diaminobenzidine tetrahydrochloride only. The specificities of the CREB antibodies have been reported previously in multiple rodent species (33) including the mouse (34). The omission of primary antibodies in these studies resulted in a complete absence of immunoreactivity. All GnRH neurons observed in the set of sections (eight to 12 sections per brain) were counted and scored for the presence or absence of CREB or pCREB. Because intensity of pCREB staining varied in different cells, only cells expressing a dark, black nuclear staining were recorded as positive.

Experiment 3: single cell RT-PCR analysis of PRL-R mRNA in GnRH neurons

Diestrous female mice expressing green fluorescent protein (GFP) under the control of the GnRH promoter (GnRH-GFP) (35) were killed between 0900 and 1200 h by cervical dislocation. As a positive control for PRL-R expression (36), dopamine neurons from the arcuate nucleus were also collected from mice expressing GFP under the control of the tyrosine hydroxylase (TH) promotor (TH-GFP) (37). The brains were rapidly removed and placed in ice-cold cutting Krebs solution [118 mM NaCl, 3 mM KCl, 0.5 mM $CaCl_2$, 6 mM $MgCl_2$, 5 mM HEPES, 25 mM $NaHCO_3$, 11 mM D-glucose (pH 7.3) when gassed with 95% O_2-5% CO_2]. Serial coronal slices (200 μm thick) were prepared using a vibratome. For GnRH neurons, sections containing the diagonal band of Broca and rostral preoptic area (rPOA) were selected, whereas for TH neurons, sections containing the arcuate nucleus of the hypothalamus were chosen. The slices were maintained in a holding chamber containing oxygenated standard Krebs solution [118 mM NaCl, 3 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5 mM HEPES, 25 mM $NaHCO_3$, 11 mM D-glucose (pH 7.3) as above] at 30 C for at least 30 min before cell harvesting at room temperature (22 C).

To harvest identified neurons, slices were placed in a recording chamber and mounted onto the stage of an upright microscope (BX51; Olympus, Tokyo, Japan) fitted with differential interference contrast optics. Slices were held submerged with weighted nylon mesh and continuously superfused with oxygenated standard Krebs at 22 C. GnRH neurons were identified as vertically oriented GFP-expressing bipolar cells located in the diagonal band of Broca and rPOA adjacent to the organum vasculosum of the lamina terminalis. GFP-labeled TH neurons were identified predominantly within the dorsomedial aspect of the arcuate nucleus, using the third ventricle and median eminence as landmarks. Patch electrodes used to harvest cell cytoplasm were fabricated from thin-walled borosilicate glass tubing (1.5 mm outer diameter; GC150TF-7.5; Harvard Apparatus, Edenbridge, UK). Electrode tubing was first baked at 250 C for 6 h before being pulled on a Flaming-Brown P-97 puller (Sutter Instruments, Novato, CA) to a tip resistance of 4–5 MΩ. Patch electrodes had resistances of 8 MΩ when filled with 8 μl of autoclaved internal solution [140 mM KCl, 10 mM EGTA, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES (pH to 7.3) with KOH]. The GnRH or TH neurons were located with fluorescence illumination to detect GFP (reflected light fluorescence illuminator BX-RFA and filter U-MWIBA2, BA510–550; Olympus) using a ×40 water immersion objective lens (LUMPlandFl, ×40, 0.80 W; Olympus). Using differential interference contrast optics, fluorescent neurons were approached with a patch pipette under positive pressure to keep slice debris from entering the pipette. After contact with the cell body, steady negative pressure was applied to the electrode to form a high resistance seal (1 GΩ), and the cytoplasm was harvested under visual control. In most cases fluorescent cytoplasm could be seen within the tip of the pipette. As a control for debris that may enter the pipette and be amplified, we conducted mock harvests in which the patch electrode was lowered into the slice, but no cellular contents were removed. All cells were harvested within 2 h of decapitation of the mouse.

Immediately after harvesting, the contents of the individual cells were expelled from the patch electrode into 8.5 μl of reverse transcriptase (RT) mixture [containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 20 mM dithiothreitol, 0.5 mM deoxynucleotide triphosphates (dNTPs), 100 ng random hexamer primers, and 200 ng oligo(dT) 12–15] and incubated at 65 C for 5 min to allow RNA denaturation and primer binding. After brief cooling, 20 U RNase inhibitor (RNaseOUT; Invitrogen, Carlsbad, CA) and 200 U Superscript III reverse transcriptase (Invitrogen) were added, and the reactions were incubated first at room temperature for 5 min and then at 50 C for 1 h for cDNA synthesis. After cDNA synthesis, enzymes were heat inactivated by incubation at 70 C for 15 min and the reactions were stored at −80 C until use (within 2 wk).

Multiplex PCR was undertaken in a manner similar to that described previously (38, 39). Gene-specific oligonucleotide primers for PCR were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) on cDNA or genomic DNA sequences representing each gene of interest (see Table 1 for GenBank accession numbers). First-round PCR was performed on 5 μl of RT product from each cell using two sets of primer pairs pooled in a 100-μl reaction containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 10 pmol of each primer (GnRH F1 and R1 or TH F1 and R1, depending on the cell type collected, and PRL-R F1 and R1; see Table 1 for sequences) and 2.5 U *Taq* polymerase (Roche Diagnostics, Auckland, New Zealand). Cell contents processed in parallel, but without RT, pipette solution from mock harvests, or water in place of template were used as negative controls. One nanogram of cDNA from whole hypothalamus was used as a positive control for all transcripts. Primers for GnRH and PRL-R were intron spanning and could be used to detect the presence of contaminating genomic DNA. Thirty-six cycles of first-round amplification were performed using a PTC-200 (MJ Research, Waltham, MA) in thin-walled, 0.2-ml PCR tubes according to the following protocol: first cycle of 95 C (3 min), 59 C (2 min), and 72 C (3 min) followed by 35 cycles of 95 C (40 sec), 59 C (1 min), and 72 C (1 min). A final 5-min incubation at 72 C was used to polish the DNA termini. Examination of first-round products was not performed on a regular basis, but in preliminary experiments developing the methodology, we could usually detect the presence of GnRH product. Second-round nested PCR was performed using 1.25-μl aliquots of the first-round amplicon pool. PCR for GnRH or TH (depending on the cell type) and PRL-R was then performed in separate 25-μl reactions containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 μM of the appropriate nested oligonucleotide primer pair (GnRH F2 and R2, TH F2 and R2, or PRL-R F2 and R2), and 0.6 U *Taq* polymerase (Roche). Thirty-six cycles of amplification were undertaken using the same protocol described for the first-round PCR except that annealing was carried out at 58 C. The resulting amplicons (GnRH 213 bp, TH 285 bp, PRL-R 305 bp) were resolved on ethidium bromide-stained 2% agarose gels in Tris-acetic acid-EDTA and photographed using a gel documentation system (GeneSnap; Syngene, Cambridge, UK).

Statistical analysis

Data are presented as mean ± SEM. LH data were analyzed using one-way ANOVA. Where the F ratio was significant, this was followed by *post hoc* analysis with Fisher's protected least significant difference test using GraphPad software (GraphPad, San Diego, CA). Percentages of GnRH neurons expressing CREB or pCREB were compared using the Kruskal-Wallis nonparametric ANOVA. If a significant H-statistic was detected, the Mann-Whitney *U* test was used to compare between groups. In all analyses, differences were considered statistically significant at $P < 0.05$.

Results

Experiment 1: effect of icv prolactin infusion on LH secretion in vivo

The OVX+E model used in these experiments resulted in basal serum LH levels of 4.9 ± 1.2 ng/ml (n = 8). Seven days infusion of oPRL into the lateral ventricle (n = 8) resulted in a significant ($P < 0.05$) suppression of serum LH levels (Fig. 1).

Experiment 2: prolactin-induced phosphorylation of CREB in GnRH neurons

Immunoreactivity for CREB and pCREB was restricted to cell nuclei and detected with a heterogeneous distribution throughout the brain. Figure 2 shows representative images of the dual-label immunohistochemistry of pCREB in GnRH neurons. Data were quantified by counting the total number of GnRH neurons expressing pCREB and expressing this as a percentage of the total number of GnRH neurons counted. The total numbers of GnRH neurons detected varied according to anatomical location, with peak numbers of 18.3 ± 1.8 GnRH cells per coronal section detected at the level of the rPOA. Neither the distribution nor total number of GnRH neurons counted was changed in any of the treatment groups (data not shown). Three independent experiments were undertaken. For the first experiment, intact diestrous mice were used. In control animals given an acute saline injection, levels of pCREB in GnRH neurons was low (5.0 ± 1.8% of GnRH neurons positive for pCREB, n = 5). Acute administration of oPRL (n = 6) resulted in an approximately 2-fold increase in the number of GnRH neurons expressing pCREB (Fig. 3A). The GnRH neurons expressing pCREB were detected throughout the continuum of GnRH neurons in the basal forebrain. In the second experiment, the same procedure was undertaken in OVX+E mice. Basal expression of pCREB was slightly higher (11.3 ± 6.3%, n = 5), and acute administration of oPRL (n = 4) resulted in a significant 2-fold increase in the number of GnRH neurons expressing pCREB (Fig. 3B). In the final experiment, a more chronic mode of prolactin administration was used, with five injections over the course of 48 h

TABLE 1. Gene names, accession numbers, primer names, and sequences

Gene name	Accession no.	Primer name	Sequence
GnRH	M14872	GnRH-F1	TGCTCCAGCCAGCACTGGTCC
		GnRH-R1	CAATGTTATACTAGGGTGTTGTGG
		GnRH-F2	CACTGGTCCTATGGGTTGCGC
		GnRH-R2	AGTGCATCTACATCTTCTTCTGCC
PRL-R[a]	NM_011169.3	PRLR-F1	TCACTCCTCTCCTGCGTTCT
		PRLR-R1	GAAGCAGTACCTCGGATCCA
		PRLR-F2	GCCATCTGCACTTGCTTACA
		PRLR-R2	CACAGTAAATGCCACGAACG
TH	NM_009377	TH-F1	GCCGTCTCAGAGCAGGATAC
		TH-R1	GGGTAGCATAGAGGCCCTTC
		TH-F2	AGGAGAGGGATGGAAATGCT
		TH-R2	ACCAGGGAACCTTGTCCTCT

[a] Primers designed to detect both long and short forms of the receptor mRNA.

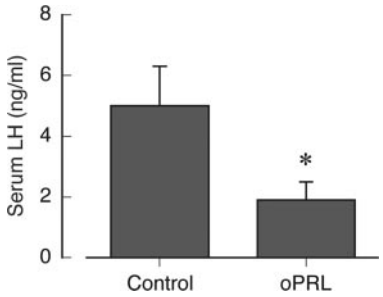

FIG. 1. Serum LH levels (mean ± SEM) in OVX+E mice, either treated with icv infusion (625 ng/h for 7 d) of oPRL or vehicle control. *, Significantly different from control ($P < 0.05$).

in OVX+E mice. Again, control animals had very low levels of pCREB expression (2.2 ± 0.3%, n = 6), whereas chronic prolactin treatment (n = 6) induced approximately 4-fold increase in the number of GnRH neurons expressing pCREB (Fig. 3C). Levels of pCREB in GnRH neurons in bromocriptine-treated animals were not significantly different from controls. In all experiments, the expression of CREB by GnRH neurons was not changed (Fig. 3, D–F).

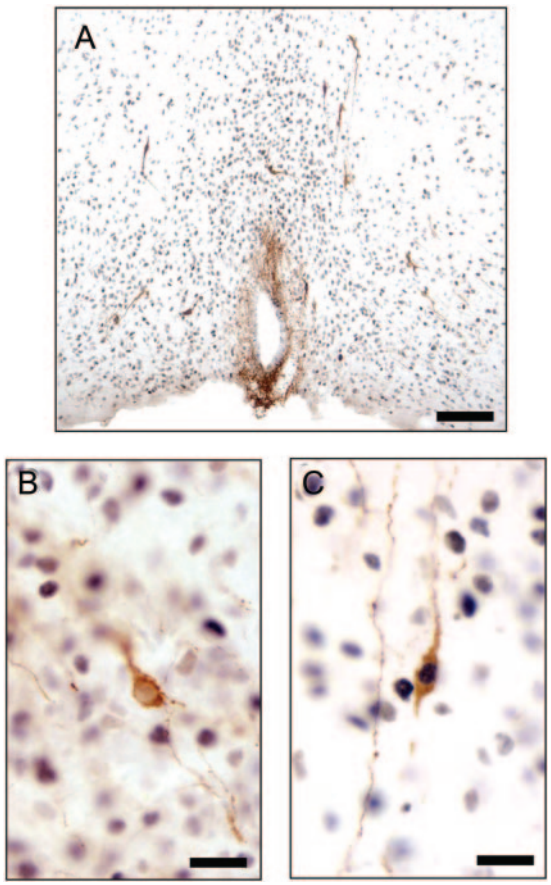

FIG. 2. Representative images of dual-label immunohistochemistry showing GnRH neurons (*brown cytoplasmic stain*) and pCREB (*black nuclei*). A, Low-power image showing typical distribution of GnRH neurons in the rostral hypothalamus at the level of the organum vasculosum of the lamina terminalis. Most GnRH neurons were negative for pCREB (B), with a small proportion showing distinct *black staining* of the nucleus identifying the presence of pCREB (C). *Scale bars*, 100 μm (A) or 10 μm (B and C).

Experiment 3: expression of PRL-R mRNA by individual GnRH neurons

We used the same single-cell RNA extraction technique used previously by our laboratory (38, 40) followed by a two-step nested PCR amplification for detection of transcripts. Representative gels are depicted in Fig. 4. A total of 40 GnRH-GFP neurons were harvested from the rPOA of four adult female mice; 25 such cells were examined for expression of GnRH and PRL-R, and 15 were used as no-RT controls. Other controls included using water in place of template or mock harvest in which the patch pipette was lowered into the slice and withdrawn. None of the controls yielded a PCR product. The majority of harvested GnRH-GFP neurons (23 of 25 cells) were shown by RT-PCR to contain transcripts for GnRH. Three of these 23 GnRH neurons (13% of the population) were found to coexpress PRL-R mRNA. As a positive control and to validate the efficiency of the PRL-R primers in this single-cell RT-PCR paradigm, we performed the analysis in parallel on TH-expressing cells from the arcuate nucleus of TH-GFP mice. A total of 14 arcuate nucleus TH-GFP neurons were harvested from one adult female mouse; 12 of these cells were examined for the expression of TH and PRL-R, and two were processed in parallel as no-RT controls. Eleven of 12 TH-GFP cells were shown by RT-PCR to contain transcripts for TH. Nine of these 11 TH cells (82%) also showed coexpression of PRL-R mRNA, whereas the controls were negative. The two cells that did not show expression of PRL-R mRNA were located in more ventrolateral regions of the arcuate nucleus.

Discussion

These data provide evidence that hyperprolactinemia suppresses LH secretion in female mice through a central nervous system site of action. Chronic prolactin administration was found to suppress LH secretion and both acute and chronic prolactin treatments were observed to alter CREB phosphorylation in a small subpopulation of GnRH neurons. Furthermore, we found evidence for the expression of PRL-R mRNA in a similarly small subpopulation of GnRH neurons. These observations are consistent with the hypothesis that prolactin inhibits LH secretion through an action mediated at least in part through the direct suppression of GnRH neurons.

Previous studies provided inconsistent data regarding the ability of prolactin to suppress of fertility in mice (26–28). Although it is well established that hyperprolactinemia causes infertility in many mammalian species, including humans, one study (26) found that high prolactin did not inhibit LH secretion in mice. This is in contrast to our present result and may result from the use of different mouse models in the studies. Although it has not been examined in mice, the PRL-R is positively regulated by estrogen in rats (41, 42), so we used an OVX mouse model in which low levels of estrogen were present, but in which LH levels were not completely suppressed. In this model, chronic icv prolactin clearly suppressed LH secretion, suggesting a central site of action for prolactin and demonstrating that the mouse is indeed like other mammals in this respect. There is considerable evidence in rats, sheep, and humans that prolactin can

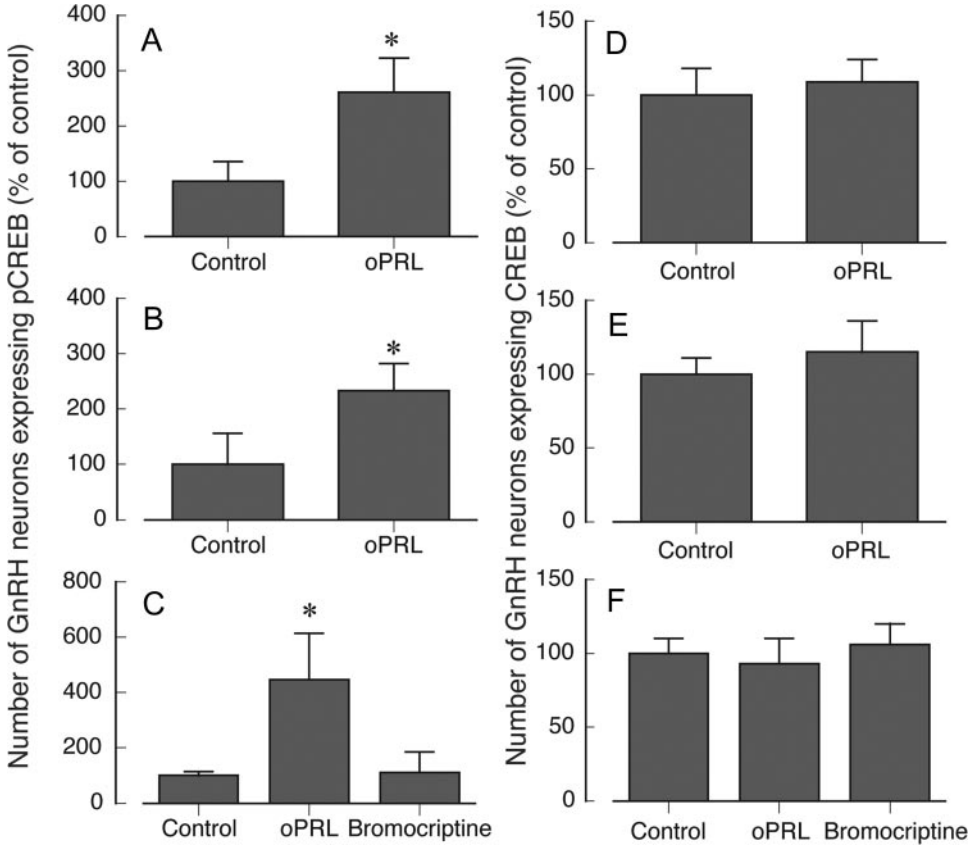

FIG. 3. Quantification of pCREB (A–C) and CREB (D–F) staining, with the percentage of pCREB- or CREB-labeled GnRH neurons in each animal depicted relative to that observed in controls (mean ± SEM). A and D, Intact, diestrous mice given acute injection of ovine prolactin (oPRL, 50 μg sc). B and E, OVX+E mice given acute injection of oPRL (50 μg sc). C and F, OVX+E mice treated twice daily for 48 h with oPRL (50 μg/injection, sc); bromocriptine (100 μg per injection, sc); or saline-vehicle controls. *, Significantly different from control ($P < 0.05$).

also act in the pituitary gland to inhibit LH secretion (43–49), and this is likely to also contribute to the infertility caused by elevated prolactin. However, the fact that hyperprolactinemia-induced infertility can be reversed by exogenous GnRH (4–6) suggests that lack of GnRH might be the primary cause of prolactin-induced infertility. At present, the effects of prolactin on mouse gonadotrophs are unknown.

Prolactin receptors are members of the cytokine receptor superfamily (50) and activate multiple signal transduction pathways. There are several isoforms of the prolactin receptor in mice: a long form and at least three short forms with truncated cytoplasmic domains (51), although only the long form is able to initiate full signaling. Prolactin binding to the long form of the receptor induces activation of the janus kinase-signal transducer and activator of transcription (STAT) pathway, in particular inducing phosphorylation of STAT5a and 5b. Phosphorylated STAT proteins translocate to the nucleus and bind to specific sequences in the promoters of target genes, regulating transcription. We have shown in both rats and mice that STAT5b is specifically required for prolactin action in hypothalamic dopamine neurons (52, 53). Prolactin can also act through either the long or short forms of the receptor to influence a range of other pathways, which may mediate rapid actions of prolactin in other neurons (50, 54). We have demonstrated that prolactin activation of tyrosine hydroxylase activity in cultured rat hypothalamic neurons involves activation of MAPK (ERK1 and ERK2), protein kinase A, protein kinase C, and calcium/calmodulin-dependent protein kinase II (54). Finally, prolactin has also been demonstrated to exert membrane effects resulting in a rapid increase in intracellular Ca^{2+} in a range of cells (20, 55–58), probably through modulation of influx of extracellular Ca^{2+} through voltage-gated calcium channels. The activation of any of these pathways would be expected to result in elevated levels of CREB phosphorylation (31). As such, we chose to examine the phosphorylation status of CREB to evaluate whether prolactin may impact intracellular signaling within GnRH neurons. Although overall levels of CREB phosphorylation were quite low under the conditions used in this study, administration of either acute or chronic prolactin to intact or OVX+E mice was found to induce CREB phosphorylation in a subpopulation (5–10%) of GnRH neurons. This result indicates that changes in circulating prolactin levels result in altered intracellular signaling within GnRH neurons. Although the effects of altered CREB phosphorylation in GnRH neurons are unknown, it is interesting to note that both prolactin (present data) and estrogen (32) enhance CREB phosphorylation in these cells and that this is associated in both cases with a reduction in plasma LH levels. There is limited direct evidence for specific effects of pCREB in the activity of GnRH neurons, although mice with neuron-specific CREB deletion are subfertile (59). Whereas it is known that pCREB can suppress gene expression (60, 61), this has not been specifically studied in adult GnRH neurons. In GT1–7 cells, activation of CREB has been associated with a suppression of GnRH mRNA expression (62), consistent with the concept that activation of CREB could inhibit activity of GnRH neurons.

The effects of prolactin on LH levels and CREB phosphorylation indicated that GnRH neurons were regulated by this

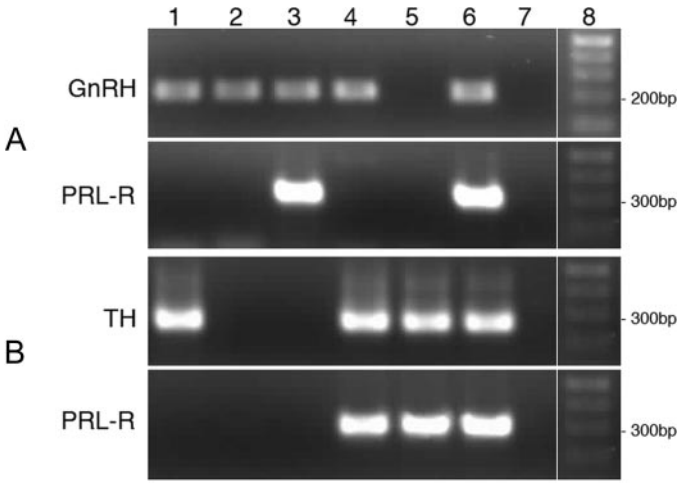

FIG. 4. Representative gels showing RT-PCR amplified transcripts from individual GFP-labeled GnRH- or TH-positive neurons. A, Cytoplasm from single GnRH-GFP neurons was harvested and subjected to RT and then PCR with gene-specific primers recognizing GnRH (A, *upper panel*, lanes 1–4) or PRL-R (A, *lower panel*, lanes 1–4). cDNA from whole medial basal hypothalamus was used as a positive control (A, *upper* and *lower*, lane 6). Negative controls, processed in parallel, included either reactions without reverse transcriptase (A, *upper* and *lower*, lane 5) or water in place of template (A, *upper* and *lower*, lane 7). Note PRL-R transcripts could be detected in a small proportion of GnRH neurons. B, Cytoplasm from single TH-GFP neurons of the arcuate nucleus was harvested and subjected to RT and then PCR with gene-specific primers recognizing TH (B, *upper panel*, lanes 1 and 4–6) or PRL-R (B, *lower panel*, lanes 1 and 4–6). Negative controls, processed in parallel, included RT reaction without reverse transcriptase enzyme (B, *upper* and *lower*, lane 2), mock harvests (B, *upper* and *lower*, lane 3), or water in place of template (B, *upper* and *lower*, lane 7). Note PRL-R transcripts could be detected in most arcuate TH neurons. Lane 8 (*all panels*) contains molecular weight markers, with sizes indicated as needed. The expected amplicon sizes are: GnRH, 215 bp, TH, 285 bp, and PRL-R, 305 bp.

hormone. To evaluate whether these regulatory actions may occur directly or indirectly on GnRH neurons, we used a now well-established and sensitive single cell RT-PCR approach (38, 40, 63) to examine for the presence of PRL-R mRNA in GnRH neurons of the adult female. The present data show that PRL-R mRNA can be detected in a small subpopulation (13%) of GnRH neurons in the mouse. At this stage, we cannot distinguish whether GnRH neurons contain the long or short forms of the PRL-R. To assess the efficiency of our single-cell RT-PCR approach for evaluating PRL-R mRNA expression, we examined expression in dopamine neurons of the arcuate nucleus, a very high proportion of which are known to express PRL-R mRNA in rats (36). We found that 82% of these dopamine neurons were positive for PRL-R mRNA, suggesting that there was no technical problem in detecting the mRNA when present. Thus, it appears that the GnRH neurons are heterogeneous with respect to PRL-R mRNA expression, and only a small subpopulation express PRL-R. This finding is similar to our preliminary work in the rat, in which we found that PRL-R mRNA (long form) could be detected in only approximately 5% of adult GnRH neurons using dual-label *in situ* hybridization (64). Such heterogeneity is common in adult GnRH neurons (65), although the functional consequences are not clear. It is tempting on the basis of the similar percentages to speculate that the subpopulation of GnRH neurons expressing PRL-R mRNA are also the ones showing pCREB expression in response to prolactin.

Although the present results provide evidence for a direct action of prolactin on GnRH neurons, it is important to recognize that indirect mechanisms may also exist. The network of neuronal inputs to GnRH neurons in the mouse has started to be unraveled (66), and prolactin receptors are known to be expressed in several areas, such as the anteroventral periventricular and arcuate nuclei (16, 67), that contain neurons directly innervating rPOA GnRH neurons. In addition, there is evidence that prolactin can regulate the activity and biosynthesis of γ-aminobutyric acid (20), opioid (21), neuropeptide Y (22), and dopaminergic (23) neuronal populations in the hypothalamus. Thus, as is apparent for the many effects of estrogen on GnRH neurons (65), the final actions of any one hormone may result from a combination of direct and indirect actions.

In conclusion, we report here that hyperprolactinemia results in the suppression of LH secretion and that this action is likely to be mediated at least in part through direct actions of prolactin on GnRH neurons. We also show that estrogen-treated ovariectomized female mice provide a suitable model for investigating the mechanisms of hyperprolactinemia-induced infertility, providing an opportunity to use transgenic models to investigate further prolactin actions on GnRH neurons in the adult brain.

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