Identification of Prolactin-Sensitive GABA and Kisspeptin Neurons in Regions of the Rat Hypothalamus Involved in the Control of Fertility

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High levels of circulating prolactin are known to cause infertility, but the precise mechanisms by which prolactin influences the neuroendocrine axis are yet to be determined. We used dual-label *in situ* hybridization to investigate whether prolactin-receptor (PRLR) mRNA is expressed in GnRH neurons. In addition, because γ-aminobutyric acidergic and kisspeptin neurons in the rostral hypothalamus are known to regulate GnRH neurons and, hence, might mediate the actions of prolactin, we investigated whether these neurons coexpress PRLR mRNA. ^{35}S-labeled RNA probes to detect PRLR mRNA were hybridized together with digoxigenin-labeled probes to detect either GnRH, *Gad1*/*Gad2*, or *Kiss1* mRNA in the rostral hypothalamus of ovariectomized (OVX), estradiol-treated rats. Additional sets of serial sections were cut through the arcuate nucleus of OVX rats, without estradiol replacement, to examine coexpression of PRLR mRNA in the arcuate population of kisspeptin neurons. PRLR mRNA was highly expressed throughout the rostral preoptic area, particularly in periventricular regions surrounding the third ventricle, and there was a high degree of colocalization of PRLR mRNA in both *Gad1*/*Gad2* and *Kiss1* mRNA-containing cells (86 and 85.5%, respectively). In contrast, only a small number of GnRH neurons (<5%) was found to coexpress PRLR mRNA. In the arcuate nucleus of OVX rats, the majority of *Kiss1* mRNA-containing cells also coexpressed PRLR mRNA. These data are consistent with the hypothesis that, in addition to a direct action on a small subpopulation of GnRH neurons, prolactin actions on GnRH neurons are predominantly mediated indirectly, through known afferent pathways. **(*Endocrinology* 152: 526–535, 2011)**

Hyperprolactinaemia is the most common disorder of the hypothalamic-pituitary axis (1) and is well recognized as a major cause of reproductive dysfunction in both males and females. Approximately 40% of women presenting with secondary amenorrhoea have increased serum prolactin levels (2, 3). Men may similarly present with infertility and decreased libido, with hyperprolactinemia present in approximately 16% of patients with erectile dysfunction and 11% of men with oligospermia (4). Infertility and loss of libido are serious side effects caused by the hyperprolactinemia induced by neuroleptic drugs, affecting up to 70% of patients and representing one of the major causes of noncompliance with antipsychotic medication (3). Further understanding of the mechanism of prolactin in suppressing reproduction may allow better therapy or drug design.

Precisely how hyperprolactinaemia disrupts the reproductive axis is unclear. In humans, elevated circulating levels of prolactin are associated with a marked reduction in both the frequency and amplitude of LH pulses (5), a direct result of GnRH pulses, and the suppression of LH pulsatility can be reversed by reducing serum prolactin concentrations to normal (6). Similarly, prolactin suppresses both the frequency and the amplitude of LH pulses

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Abbreviations: E, Estradiol; GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase; OVX, ovariectomized; PRLR, prolactin receptor; RP3V, rostral periventricular area of the third ventricle; TIDA, tuberoinfundibular dopamine.

in rats (7–10), and direct measurements of GnRH secretion into the portal blood have revealed a prolactin-induced suppression of GnRH release (11, 12). Furthermore, during lactation, a physiological state characterized by high levels of circulating prolactin, pulsatile LH release is suppressed (13, 14), and the activity of GnRH neurons is reduced (15). Although there is evidence that prolactin can also act in the pituitary gland to suppress LH and FSH secretion (16–18), pulsatile GnRH replacement can reverse the infertility induced by hyperprolactinaemia (19, 20), suggesting that prolactin-induced suppression of GnRH release is the principal cause of infertility.

We recently reported that a small proportion of GnRH neurons in mice express the prolactin receptor (PRLR) and show prolactin-induced changes in cAMP response element-binding protein phosphorylation (21). These data suggested that prolactin might act directly on some GnRH neurons to regulate fertility. The infrequent expression of PRLRs in GnRH neurons, however, contrasts markedly with the relatively high levels of PRLR expression in regions of the rostral hypothalamus known to be involved in the control of GnRH secretion (22, 23). The majority of GnRH neuronal cell bodies reside in the preoptic area/anterior hypothalamus and the hypothalamic region extending from the anteroventral periventricular nucleus to the periventricular preoptic region [collectively designated the rostral periventricular area of the third ventricle (RP3V) (24)] is well established to be involved in the regulation of GnRH neurons (24–27). The RP3V also contains abundant PRLR mRNA (22, 23), suggesting that prolactin actions on GnRH neurons could be mediated indirectly through actions of RP3V neuronal populations. The neurochemical phenotype of the PRLR-expressing neurons in the RP3V, however, is not known.

Based on the current literature, the two most likely populations in the RP3V that could be involved in regulating GnRH neuronal activity are γ-aminobutyric acid (GABA)ergic and kisspeptin neurons. There is convincing evidence that the inhibitory neurotransmitter GABA is an important player in the regulation of GnRH secretion (28; also see reviews in Refs. 29, 30). GABAergic neurons form synapses onto GnRH neurons (31), and studies using *in situ* hybridization (32, 33) or single cell RT-PCR methodology (34) have shown that GnRH neurons express mRNA for GABA receptor subunits. In addition, a wealth of electrophysiological data has shown that GABA affects the activity of GnRH neurons (35–38). Neurons expressing the neuropeptide kisspeptin are also found prominently in the RP3V (39). GnRH neurons express G protein-coupled receptor 54, the kisspeptin receptor (40–42), and kisspeptin potently activates GnRH activity (40, 42, 43). In female rodents, kisspeptin is one of the essential components mediating the onset of GnRH activity at puberty [reviewed by Clarkson *et al.* (44)] and plays a role in inducing the preovulatory GnRH surge (43, 45, 46).

We hypothesize that prolactin may influence GnRH neurons indirectly, through actions on afferent GABA and kisspeptin neuronal populations located in the RP3V. The aim of this study was to use dual-label *in situ* hybridization to determine whether these neuronal populations express PRLR mRNA. In addition, because there is a second population of kisspeptin neurons present in the arcuate nucleus of the rodent hypothalamus (43, 47–49), and this nucleus contains many PRLR-expressing neurons (22, 23, 50–52), we also investigated PRLR expression in the arcuate kisspeptin neurons. Finally, to extend data from mice showing expression of PRLRs in a subpopulation of GnRH neurons, we examined whether GnRH neurons in the rat expressed PRLR (long form) mRNA.

Materials and Methods

Animals and tissue preparation

All animal manipulations were approved by the University of Otago Animal Ethics committee. Virgin, adult female Sprague Dawley rats (10–12 wk old) were group housed under standard, controlled laboratory conditions (14-h light, 10-h dark cycle; temperature, 21 ± 1 C). Rats were bilaterally ovariectomized (OVX) under halothane anesthesia using sterile surgical procedures. Seven days later, silastic implants (length, 30 mm; inside diameter, 1.57 mm; outside diameter, 3.18 mm) containing either 150 μg/ml of 17β-estradiol (E) to restore low physiological levels (equivalent to diestrus) (OVX+E) or vehicle (sesame oil) were implanted sc. This level of E is not sufficient to produce a daily LH or prolactin surge. One week later, between 0930 and 1100 h, all rats were anesthetized with an overdose of sodium pentobarbitone, then transcardially perfused with buffered 2% paraformaldehyde. Brains were removed, postfixed overnight, then cryoprotected in 30% sucrose in phosphate buffer for 72 h before being frozen on powdered dry ice and stored at −80 C. Sets of serial, coronal sections of 16-μm thickness were cut through the rostral hypothalamus from the preoptic region to the periventricular area (bregma 0.00 to −0.84 mm) and through the arcuate nucleus (bregma −2.10 to −3.30 mm) (53) and mounted onto aminopropyltriethoxysilane-coated slides. Sections were stored at −80 C until used for *in situ* hybridization.

Preparation of probes for *in situ* hybridization

To prepare probes to detect *Kiss1* and *Gad1/Gad2*, genes encoding kisspeptin-54 and glutamic acid decarboxylase (GAD)1 and GAD2, respectively, and PRLR mRNA, cDNA templates were first generated by RT-PCR. Primer pairs were designed using sequences from GenBank for the long form of the PRLR (accession no. M57668), for *Kiss1* (accession no. NM 181692), and for *Gad2* and *Gad1* (accession nos. NM012563 and NM 1077007, respectively), and these primer pairs used to generate cDNA templates (290–313 bp) that incorporated T7 and SP6 RNA polymerase sequence sites. The specificity of all the cDNA templates was confirmed by sequencing. ^{35}S-labeled

cRNA probes targeted to the PRLR were generated using T7 polymerase (antisense probe) or SP6 polymerase (sense probe) in accordance with the Riboprobe In Vitro kit (Promega, Madison, WI) protocol as described previously (51). Antisense and sense cRNA probes for *Kiss1*, *Gad1*, and *Gad2*, labeled with digoxigenin, were transcribed from the cDNA templates using a digoxigenin RNA-Labeling kit (Roche Diagnostics GmbH, Mannheim, Germany) and the appropriate polymerases. A cRNA probe targeted to GnRH mRNA was transcribed from a 330 bp *Bam*H1-*Hin*d111 cDNA construct subcloned into a p65T7 vector. The cDNA was linearized with *Bam*H1, then transcribed with T7 polymerase in the presence of digoxigenin uradine triphosphate, using a commercial digoxigenin RNA-Labeling kit (Roche). Unincorporated nucleotides were removed from all labeled probes with mini Quick Spin RNA purification columns (Roche).

Dual-label *in situ* hybridization histochemistry

Sections were pretreated with a 5 min, 2% paraformaldehyde fixation step, permeabilized with proteinase K, then acetylated and prehybridized in the hybridization solution [100 mM dithiothreitol, 0.3 M NaCl, 20 mM Tris (pH 8), 5 mM EDTA (pH 8), 1× Denhardt's solution, 10% dextran sulfate, and 50% formamide] without the labeled cRNA probes for 1–3 h. The probes were denatured at 95 C for 3 min, then diluted into hybridization solution, pipetted onto the sections, and the slides were coverslipped (Hybri-slips; Sigma-Aldrich, St. Louis, MO). Sections were hybridized for 16 h at 55 C with an ^{35}S-labeled RNA probe (1.2×10^6 cpm per 120 μl of hybridization solution) complementary to the long form of the PRLR together with digoxigenin-labeled probes (0.5 ng/μl·Kb per section) specific for either GnRH, *Gad1* and *Gad2*, or *Kiss1* mRNA. After hybridization, sections were washed in sodium saline citrate solution (150 mM NaCl and 15 mM sodium citrate) containing ß-mercaptoethanol, subjected to ribonuclease treatment, followed by a series of washes (most stringent, 0.1 M sodium saline citrate at 55 C with shaking for 2 h). To visualize the digoxigenin-labeled mRNA hybrids, sections were incubated in sheep antidigoxigenin antibody conjugated to alkaline phosphatase (1:2000 dilution) for 24 h at 4 C, then detected using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as a substrate. After air-drying, slides were dipped in undiluted LM-1 Hypercoat emulsion (Amersham Pharmacia Biotech, Piscataway, NJ), sealed in lightproof boxes with desiccant and stored at 4 C for 4–5 wk. Slides were developed with D-19 developer (Kodak, New York, NY), fixed with Ilford Hypan fixer, dehydrated through alcohols, cleared in xylene, then mounted with VectaMount mounting medium, and coverslipped. To generate autoradiograms, the majority of slides were also apposed to film (Kodak BioMax MR) for 3–5 d before emulsion coating.

Data analysis

Sections were photographed under brightfield illumination with ×40 objective, using a SPOT RT digital camera and associated SPOT software (Diagnostic Instruments, Inc., Sterling Heights, MI), attached to an Olympus BX51 microscope. Digoxigenin-labeled cells were identified by the presence of a distinct blue/purple cytoplasmic precipitate. For GnRH neurons, all digoxigenin-labeled GnRH mRNA-positive cells were counted in five to seven sections throughout the preoptic area and rostral hypothalamus of OVX, E-treated rats. Data are presented as the total number counted, per brain (n = 4, mean ± SEM). For *Kiss1* and *Gad1*/*Gad2* digoxigenin-labeled mRNA-positive cells, a single field of view was photographed at ×400 magnification on each side of the brain, using the third ventricle to define one edge of the field. All labeled cells within these fields were counted in each of four sections through the RP3V region of the hypothalamus of OVX+E rats (n = 4). In the arcuate nucleus, all *Kiss1* mRNA-positive cells were counted in four to six slides from both OVX and OVX+E rats (n = 4 in each group). For *Kiss1* and *Gad1*/*Gad2* mRNA, data are expressed as the mean number of identified cells per section in each brain (mean ± SEM).

To assess the number of PRLR-expressing cells, the number of silver grains over each cell (proportional to the number of PRLR mRNA hybrids) in these same sections was quantified using ImageJ software (National Institutes of Health). Silver grains were identified and analyzed under brightfield illumination and confirmed by darkfield inspection of the sections. Mean background levels of silver grains on each slide were sampled from five random areas, which were approximately equivalent in size to that of the silver grain clusters present over neuronal cell bodies. PRLR-positive cells were defined as those in which the density of clusters of silver grains overlying neuronal cell bodies was greater than three times background silver grain levels. Cells were counted as double labeled if a digoxigenin-labeled cell was also positive for PRLR mRNA. Differences between groups were calculated by ANOVA followed by Newman-Keuls *post hoc* tests using GraphPad Prism software for Macintosh (GraphPad, San Diego, CA). The level of significance was set at $P < 0.05$.

Results

Double-label *in situ* hybridization for PRLR mRNA and GnRH mRNA

As shown in the autoradiograph and associated darkfield image in Fig. 1, A and B, high levels of PRLR-expressing cells were found throughout the RP3V region, particularly in the area close to the third ventricle. A representative image of cells subjected to double-label *in situ* hybridization experiments to detect colocalization of PRLR and GnRH mRNA expression is shown in Fig. 1C. Neuronal cell bodies expressing GnRH mRNA hybrids, visible as a colored precipitate in the cytoplasm of the cell, were detected in a scattered pattern throughout the rostral preoptic area. This pattern of expression closely matches the well-established distribution of GnRH neurons (54), thus confirming the specificity of the digoxigenin-labeled probe. Although PRLR mRNA transcripts were abundant in the RP3V region of the hypothalamus and in the adjacent medial preoptic area (particularly in the ventromedial preoptic nucleus, which is sparsely populated with GnRH neurons), only a few GnRH neurons (≈5%) were found to coexpress PRLR mRNA, evidenced by the lack of visible silver grain clusters over GnRH neuronal cell bodies (Fig. 1, C and D).

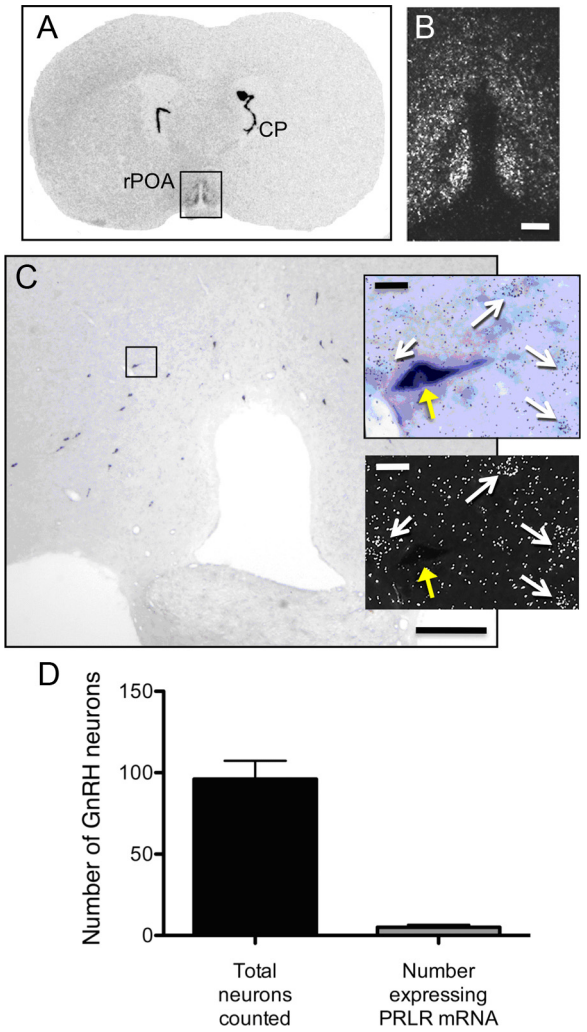

FIG. 1. Dual labeling of GnRH and PRLR mRNA by *in situ* hybridization in the rostral preoptic area. A, Representative autoradiogram showing PRLR mRNA (*black areas*) in the rostral preoptic area (note also strong labeling in the choroid plexus). rPOA, Rostral preoptic area; CP, choroid plexus. B, Darkfield image of the *box* shown in A with PRLR mRNA revealed as white clusters of silver grains. C, Micrograph showing localization of GnRH neurons (blue/purple cytoplasmic precipitate) and PRLR mRNA (clusters of black grains). Section has been counterstained with Gills hematoxylin to identify cell nuclei. *Insets*, Brightfield (*upper*) and darkfield (*lower*); high-power views of the area in the *box* of C. *White arrows* indicate PRLR mRNA-positive cells, visualized as clusters of silver grains. *Yellow arrow* indicates a GnRH neuron. Note absence of silver grains over this cell, illustrating lack of colocalization of GnRH and PRLR mRNA. D, Histogram comparing the mean number of GnRH neurons counted per rat with the mean number of GnRH neurons expressing PRLR mRNA. *Scale bar*, 100 μm (B and C) and 10 μm (*insets*).

Colocalization of PRLR mRNA and *Gad1/Gad2* mRNA

GABAergic neurons were detected using a mixture of two probes that were targeted to the two genes (*Gad1* and *Gad2*), which encode the GABAergic synthesizing enzyme, glutamatic acid decarboxylase. In the rat brain, almost all GABAergic neurons express both *Gad1* and *Gad2* mRNA (55, 56.) Results from dual-label *in situ* hybridization experiments for PRLR mRNA labeled with ^{35}S together with GABAergic neurons visualized using a mixture of the two digoxigenin-labeled cRNA probes are illustrated in Fig. 2. As has been characterized previously (57, 58), there was a large number of GABAergic neurons present throughout the RP3V. A high degree of colocalization of PRLR mRNA in *Gad1/Gad2* mRNA-containing cells was observed as shown by the presence of dense clusters of silver grains over individual *Gad1/Gad2*-digoxigenin-labeled cell soma (Fig. 2C). Over 85% of PRLR mRNA-positive cells expressed *Gad1/Gad2* mRNA, suggesting that the vast majority of these cells are GABAergic (Fig. 2D). This high level of coexpression was observed throughout the RP3V region as well as in the

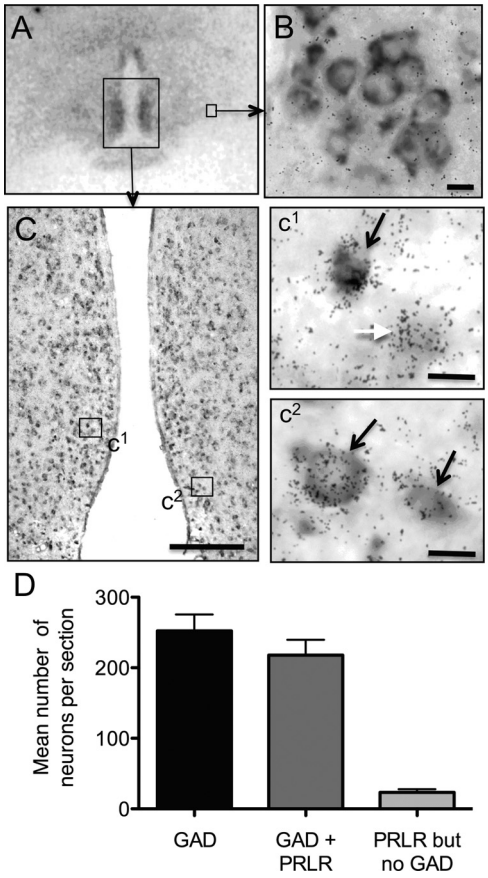

FIG. 2. Coexpression of *Gad1/Gad2* (GAD) and PRLR mRNA in the RP3V region of the hypothalamus. A, Representative autoradiogram showing localization of PRLR mRNA (*black areas*) in the periventricular region. B, Most GAD-positive cells outside the RP3V do not colocalize with PRLR mRNA, illustrated in this example from the horizontal limb of the diagonal band of Broca. C, Low-power view showing localization of GAD mRNA and PRLR mRNA in the RP3V region. *Insets* c^1 and c^2 are high-power photomicrographs of *boxes* in C, showing examples of GAD mRNA-containing cells coexpressing PRLR mRNA in the RP3V region. *Black arrows* indicate double-labeled cells, whereas the *white arrow* indicates a non-GABAergic cell single labeled for PRLR mRNA. D, Histogram comparing the mean number of GAD neurons counted (average per section) with the mean number that coexpressed prolactin mRNA. Note that relatively few neurons that expressed PRLR were not also GAD positive. *Scale bar*, 100 μm (C) and 10 μm (B, c^1, and c^2).

medial preoptic area, but was specific to these periventricular regions. In other brain regions present in the same sections, where GABA cells were also numerous, for example, the horizontal limb of the diagonal band of Broca, *Gad1/Gad2* mRNA-containing cells did not coexpress PRLR mRNA (Fig. 2B).

Colocalization of PRLR mRNA and *Kiss1* mRNA

1) RP3V Kiss1 *population*

In the OVX+E rats, the pattern of *Kiss1* mRNA-expressing neurons in the RP3V was consistent with the expression patterns in rat brain reported elsewhere (59), with the highest number of *Kiss1*-expressing cells present more caudally in the periventricular area of the RP3V region (Fig. 3A). Throughout the RP3V, a high degree of colocalization of PRLR mRNA with *Kiss1* mRNA was detected with PRLR mRNA coexpressed by 86% of kisspeptin neurons (Fig. 3B). Consistent with the pattern of PRLR mRNA seen in the dual-label *Gad1/Gad2 in situ* hybridization experiments, PRLR mRNA was not restricted to the kisspeptin cells but was abundantly expressed on cells throughout the RP3V. Levels of *Kiss1* mRNA in the group that were OVX but not given E implants were virtually undetectable.

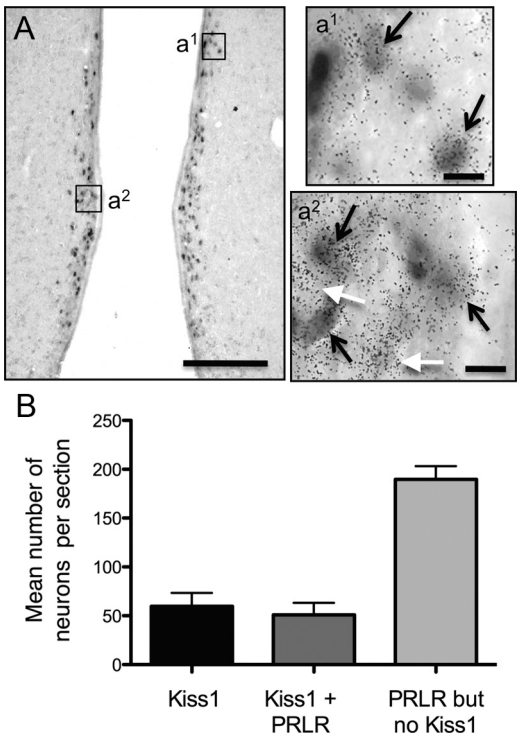

FIG. 3. Coexpression of *Kiss1* and PRLR mRNA in the RP3V region of the hypothalamus. A, Low-power view showing distribution of *Kiss1* mRNA and PRLR mRNA in the periventricular region. a^1 and a^2, High-power photomicrographs of *boxes* in A showing examples of *Kiss1* mRNA-containing cells coexpressing PRLR mRNA. *Black arrows* indicate double-labeled cells, whereas *white arrows* indicate a cell single labeled for PRLR mRNA. B, Histogram comparing the mean number of *Kiss1*-positive neurons counted (average per section) with the mean number that coexpressed prolactin mRNA. In this region, there was also a large number of PRLR-containing cells that did not express *Kiss1* mRNA. Scale bar, 100 μm (A) and 10 μm (a^1 and a^2).

2) Arcuate nucleus Kiss1 *population*

Because *Kiss1* mRNA expression in the arcuate nucleus is markedly increased when levels of circulating E are low, PRLR expression in identified kisspeptin neurons was evaluated in sections from both the OVX rat group that received oil implants as well as those from OVX+E rats. Figure 4, A and B, compares the relative distribution of *Kiss1* mRNA-positive cells (brightfield) with PRLR mRNA (darkfield) in the same section. Note that there was extensive expression of PRLR in the arcuate nucleus in the dorsomedial region, distinct from the distribution of kisspeptin cells. Analysis of the dual-label experiment shows that, although significantly more *Kiss1*-containing cells were detected in the OVX group, numerous *Kiss1* cells were also clearly detectable in the steroid-replaced group. In the former group, the majority (79%) of kisspeptin neurons was found to coexpress prolactin mRNA (Fig. 4, D and E). In OVX rats given E replacement, many fewer kisspeptin cells were detected, and a smaller proportion of those cells was double labeled (45%) (Fig. 4, C and E).

Discussion

In the present study, we have documented high levels of PRLR expression in regions of the hypothalamus known to be involved in the regulation of GnRH neurons. Consistent with our previous data in mice (21), we found that a relatively small proportion of GnRH neurons express the long form of the PRLR, but that levels of PRLR expression were high in regions of the RP3V surrounding GnRH neurons. Within the RP3V, almost all GABAergic neurons coexpressed PRLR mRNA. Similarly, many kisspeptin neurons in both the RP3V and arcuate nucleus were found to contain PRLR mRNA. These data provide new insights into potential mechanisms by which prolactin might influence GnRH neurons, suggesting an indirect pathway mediated by both stimulatory and inhibitory afferent neurons.

Our data documenting PRLR mRNA expression in the rostral hypothalamus show a relatively restricted distribution of the receptor in the RP3V region of the rostral forebrain. These data are consistent with a range of previous *in situ* hybridization studies in both rats (22, 60) and mice (23). Our autoradiographs were exposed for a relatively short period; and hence, we did not see expression in regions such as the bed nucleus of the stria terminalis and medial septum, in which PRLR mRNA could be de-

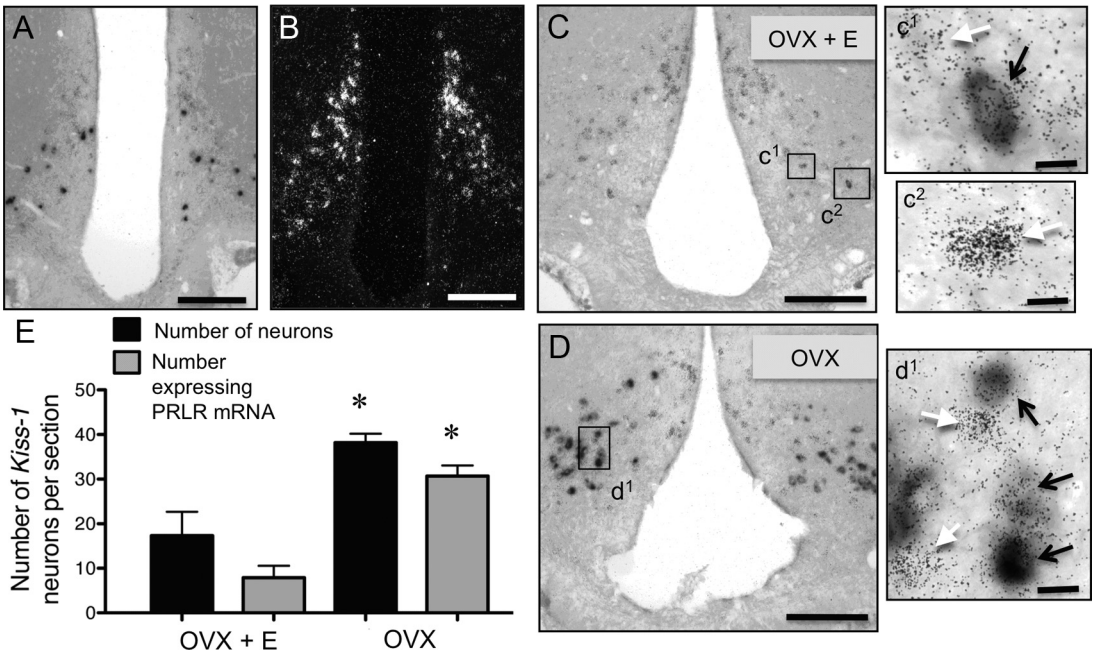

FIG. 4. Coexpression of *Kiss1* and PRLR mRNA in the arcuate nucleus of the hypothalamus. A, A coronal section at the level of the arcuate nucleus photographed under both brightfield and (B) darkfield illumination. The brightfield micrograph shows the distribution of *Kiss1* mRNA detected using a digoxigenin-labeled probe (blue/purple cytoplasmic precipitate) and PRLR mRNA (clusters of black grains). The darkfield view illustrates the distribution of PRLR mRNA as clusters of black grains overlying cells. Note that much of the PRLR mRNA in the arcuate nucleus is not associated with kisspeptin neurons. C, Low-power micrograph showing distribution of *Kiss1* mRNA and PRLR mRNA in an OVX rat given E replacement (OVX+E). c^1 and c^2, High-power photomicrographs of *boxes* in C showing examples of *Kiss1* mRNA-containing cells coexpressing PRLR mRNA. *Black arrow* indicates a double-labeled cell, whereas *white arrows* indicate cells single labeled for PRLR mRNA. D, Low-power micrograph showing distribution of *Kiss1* mRNA and PRLR mRNA in an OVX rat. d^1, High-power photomicrograph of *box* in D showing examples of *Kiss1* mRNA-containing cells coexpressing PRLR mRNA. The *black arrows* indicate double-labeled cells, whereas *white arrows* indicate cells single labeled for PRLR mRNA. E, The mean number of *Kiss1*-positive neurons that coexpressed prolactin mRNA in OVX and OVX+E-replaced rats. *, $P < 0.05$. *Scale bar*, 100 μm (A–D) and 10 μm (c^1, c^2, and d^1).

tected in mice (23). This observation suggests that levels of PRLR mRNA are higher in the periventricular areas than in these other regions and that nuclei in these regions are highly prolactin sensitive.

By far the majority of neurons in the prolactin-sensitive regions of the RP3V area is GABAergic and/or glutamatergic (61, 62). There is a large body of evidence suggesting that GABA regulates GnRH neuronal activity. GABA neurons synapse directly on GnRH neurons (31), and GnRH neurons express mRNA for several subunits of the $GABA_A$ (32) and $GABA_B$ receptor (63). Because over 85% of the GABAergic neurons in this region express PRLR mRNA, it seems highly likely that at least some of these prolactin-responsive neurons will be directly innervating GnRH neurons. Pharmacological evidence consistently demonstrates an inhibitory influence over GnRH mRNA and GnRH and LH secretion (64, 65). Although there is some controversy (66, 67), electrophysiological evidence suggests that GABAergic neurons maintain a tonic inhibitory regulation of excitability of adult GnRH neurons (36, 65). GABA also alters intracellular calcium specifically in GnRH neurons (68, 69). Hyperprolactinaemia has been reported to increase the activity of hypothalamic GABAergic neurons (70–73). We have also described increased activity of GABA neurons during lactation (74), when prolactin levels are elevated, and GABA levels in the cerebrospinal fluid are increased during lactation (75). These observations, together with the present data showing specific expression of PRLRs in GABAergic neurons in the RP3V, support the hypothesis that GABAergic neurons may mediate the suppressive effects of prolactin on GnRH neurons.

In recent years, there has been an explosion of interest in the role of kisspeptin in regulating GnRH neurons. Consistent with earlier reports (49, 59, 76), we identified two populations of kisspeptin neurons in the rodent that are reciprocally regulated by E: one population that is present in the RP3V and a second population in the arcuate nucleus. In OVX rats, kisspeptin mRNA was prominent in the arcuate nucleus, whereas there was a marked decrease in kisspeptin mRNA levels detected after E treatment. In contrast, in the RP3V, kisspeptin mRNA was undetectable in OVX rats but, after E treatment, was readily detectable. In both populations of kisspeptin neurons, a high proportion of neurons expressed PRLR mRNA, suggesting that they may be regulated by prolactin.

Kisspeptin neurons in the RP3V region project directly to the cell bodies of GnRH neurons (76) are activated by

elevated E and are essential for the generation of the preovulatory GnRH surge (43, 45, 46, 77). Thus, the presence of PRLR mRNA in 85% of these cells suggests that prolactin-responsive neurons will directly innervate GnRH neurons. These data are consistent with the hypothesis that PRLR-mediated signaling might play a role in decreasing kisspeptin levels, contributing to infertility. Interestingly, in the RP3V, there were very few PRLR-positive cells that were not also labeled with *Gad1/Gad2* mRNA, suggesting that the vast majority of these cells is GABAergic. The colocalization of some of these cells with kisspeptin, therefore, raises the interesting possibility that at least some RP3V kisspeptin neurons might coexpress GAD. Further work will be required to confirm this.

Although evidence linking RP3V kisspeptin neurons to GnRH secretion is strong, the role of the arcuate kisspeptin neurons is less clear. It has been suggested that this population of kisspeptin cells may be involved in the negative feedback regulation of GnRH secretion (43, 49), although the anatomical evidence linking these cells to GnRH neurons in rodents is still a little thin. In the monkey, kisspeptin fibers have been identified in the median eminence, closely associated with the GnRH axons and axon terminals (78), and there are some data in rodents suggesting that kisspeptin-expressing neuronal fibers, identified by coexpression of prodynorphin and proneurokinin B, terminate near GnRH fibers in the median eminence (79). It has been proposed that the arcuate population of kisspeptin neurons stimulate tonic pulsatile secretion of GnRH and are a potential site of E negative-feedback suppression of GnRH secretion (80, 81). Prolactin actions on either population of kisspeptin neurons, therefore, might influence fertility by indirectly regulating GnRH neurons. During lactation, when prolactin levels are markedly elevated, levels of *Kiss1* mRNA in the arcuate nucleus are markedly suppressed (82, 83). This is particularly interesting, because E levels are extremely low at this time, which would normally favor elevated kisspeptin expression.

It is possible that the arcuate kisspeptin neurons play other modulatory roles, rather than the control of reproduction. There is now good evidence that kisspeptin cells in the arcuate nucleus coexpress both dynorphin and neurokinin B (80, 84), and earlier studies have shown that dynorphin cells project to the tuberoinfundibular dopamine (TIDA) neurons within the arcuate nucleus (85). Changes in TIDA neuronal activity are the most important factors maintaining homeostasis of prolactin levels in the blood. There is good evidence that endogenous opioid peptides modulate TIDA neuronal activity (86–88), particularly through both κ and μ receptors (87–89), and dynorphin may play a critical role here. There is also recent evidence that kisspeptin might suppress TIDA neuronal activity (90). Hence, it is possible that prolactin-mediated signaling in the arcuate population of kisspeptin neurons might be involved in regulation of its own secretion.

The present study has shown that in hypothalamic nuclei involved in the control of fertility, both GABA and kisspeptin neurons express PRLR mRNA. In contrast, very few GnRH neurons express PRLR mRNA. These data are consistent with the hypothesis that prolactin actions on GnRH neurons are predominantly mediated indirectly through these known afferent pathways. Although hyperprolactinemia-induced infertility can be thought of as a pathological condition, arising, for example, from a pituitary adenoma, the mechanism for prolactin to suppress GnRH neurons has likely evolved as an adaptive response to pregnancy and lactation (91). Hyperprolactinemia during these physiological states is likely to contribute to the suppression of GnRH neurons that occurs in these states (13–15). This lactational infertility plays a critical role in birth spacing, ensuring optimal survival of mammalian young by minimizing maternal investment in a subsequent pregnancy (92).

Acknowledgments

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