Kisspeptin Regulates Prolactin Release through Hypothalamic Dopaminergic Neurons

Raphael E. Szawka,* Aline B. Ribeiro,* Cristiane M. Leite, Cleyde V. V. Helena, Celso R. Franci, Greg M. Anderson, Gloria E. Hoffman, and Janete A. Anselmo-Franci

Laboratório de Neuroendocrinologia (R.E.S., A.B.R., C.M.L., C.V.V.H., J.A.A.-F.), Departamento de Morfologia, Estomatologia e Fisiologia, Faculdade de Odontologia de Ribeirão Preto, 14040-904, Departamento de Fisiologia (C.R.F.), Faculdade de Medicina de Ribeirão Preto, 14049-900, Universidade de São Paulo (USP), Ribeirão Preto, São Paulo, Brazil; Centre for Neuroendocrinology and Department of Anatomy and Structural Biology (G.M.A.), School of Medical Sciences, University of Otago, 9054 Dunedin, New Zealand; and Department of Biology (G.E.H.), Morgan State University, Baltimore, Maryland 21251

Prolactin (PRL) is tonically inhibited by dopamine (DA) released from neurons in the arcuate and periventricular nuclei. Kisspeptin plays a pivotal role in LH regulation. In rodents, kisspeptin neurons are found mostly in the anteroventral periventricular and arcuate nuclei, but the physiology of arcuate kisspeptin neurons is not completely understood. We investigated the role of kisspeptin in the control of hypothalamic DA and pituitary PRL secretion in adult rats. Intracerebroventricular kisspeptin-10 (Kp-10) elicited PRL release in a dose-dependent manner in estradiol (E2)-treated ovariectomized rats (OVX+E2), whereas no effect was found in oil-treated ovariectomized rats (OVX). Kp-10 increased PRL release in males and proestrous but not diestrous females. Associated with the increase in PRL release, intracerebroventricular Kp-10 reduced Fos-related antigen expression in tyrosine hydroxylase-immunoreactive (ir) neurons of arcuate and periventricular nuclei in OVX+E2 rats, with no effect in OVX rats. Kp-10 also decreased 3,4-dihydroxyphenylacetic acid concentration and 3,4-dihydroxyphenylacetic acid-DA ratio in the median eminence but not striatum in OVX+E2 rats. Double-label immunofluorescence combined with confocal microscopy revealed kisspeptin-ir fibers in close apposition to and in contact with tyrosine hydroxylase-ir perikarya in the arcuate. In addition, Kp-10 was not found to alter PRL release from anterior pituitary cell cultures regardless of E2 treatment. We provide herein evidence that kisspeptin regulates PRL release through inhibition of hypothalamic dopaminergic neurons, and that this mechanism is E2 dependent in females. These findings suggest a new role for central kisspeptin with possible implications for reproductive physiology. **(*Endocrinology* 151: 3247–3257, 2010)**

Prolactin (PRL) is produced by the lactotrophs in the anterior pituitary gland and was initially recognized for promoting the growth of pigeon crop sac and lactation in rabbits (1). In addition to its role in lactation, PRL exerts a wide range of actions. PRL is known to regulate reproductive behaviors and to modulate the hypothalamus-pituitary-gonadal (HPG) axis (2, 3). Accordingly, the null mutation of PRL receptor in female mice results in reduced ovulation and impaired fertilization, preimplantation development, and implantation (2).

PRL secretion is tonically inhibited by dopamine (DA) released from hypothalamic neurons (3). Three distinct neuronal populations provide DA to the pituitary gland: the tuberoinfundibular dopaminergic (TIDA) neurons

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2009-1414 Received December 4, 2009. Accepted March 26, 2010.
First Published Online April 21, 2010
* R.E.S. and A.B.R. contributed equally.

Abbreviations: ARC, Arcuate; AUC, area under curve; AVPV, anteroventral periventricular; bw, body weight; C-ARC, caudal ARC; DA, dopamine; DAB, 3,3′-diaminobenzidine; DM, dorsomedial; DOPAC, 3,4-dihydroxyphenylacetic acid; E2, estradiol; FRA, Fos-related antigen; HPG, hypothalamus-pituitary-gonadal; icv, intracerebroventricular; ir, immunoreactive; Kp-10, kisspeptin-10; ME, median eminence; OVX, ovariectomized; Pe, periventricular; PRL, prolactin; R-ARC, rostral ARC; STR, striatum; TH, tyrosine hydroxylase; TIDA, tuberoinfundibular dopaminergic; Veh, vehicle; VL, ventrolateral.

that are located in the dorsomedial (DM) portion of arcuate (ARC) nucleus and project to the median eminence (ME) (4), the tuberohypophyseal dopaminergic neurons that arise from the rostral ARC and project to intermediate and neural lobes of the pituitary (5), and the periventricular hypophyseal dopaminergic neurons that arise in the periventricular (Pe) nucleus and innervate exclusively the intermediate lobe (6). Distinct patterns of PRL secretion, such as those elicited by suckling, estradiol (E2), or mating, involve inhibition of hypothalamic dopaminergic neurons as a common underlying mechanism (7). However, the nature and function of the neuronal network controlling the activity of hypothalamic dopaminergic neurons are still poorly understood.

The *KiSS1* gene encodes for a family of structurally related peptides collectively called kisspeptin, which are the natural ligands of the KiSS1R, also referred to as G protein-coupled receptor 54 (8, 9). Kisspeptin-KiSS1R signaling has been shown to be essential for the regulation of the HPG axis. Disrupted KiSS1R signaling prevents the onset of puberty in mice and humans (10), and kisspeptin has been shown to potently elicit LH secretion through activation of GnRH neurons (11–15). In rodents, *KiSS1*-expressing neurons are found mainly in the anteroventral periventricular (AVPV) and ARC nuclei. Whereas gonadal steroids stimulate *KiSS1* mRNA expression in the AVPV, they inhibit it in the ARC (16–18). This differential regulation has supported the hypothesis of two functionally distinct populations of kisspeptin neurons, *i.e.* the AVPV neurons implicated in the ovarian-steroid positive-feedback, and the ARC neurons conveying the negative-feedback actions of gonadal steroids on LH secretion (19).

Despite the role played by ARC kisspeptin neurons in the negative-feedback mechanism, the physiology of this neuronal population remains to be better elucidated. In rats, *KiSS1* expression in the ARC, but not AVPV, has been shown to be decreased during lactation (20), a physiological state of hyperprolactinemia. ARC kisspeptin neurons in ewes and mice have been shown to coexpress dynorphin and neurokinin B (21, 22). Notably, dynorphin terminals are known to display synaptic contacts with TIDA perikarya (23), with functional relevance for PRL secretion (24, 25). Thus, emerging evidence calls attention to an as yet unrevealed role for ARC kisspeptin in the regulation of TIDA neurons and PRL release. The few studies investigating kisspeptin role in PRL regulation so far have yielded conflicting results. Kisspeptin-10 (Kp-10) has been reported to not alter PRL secretion in adult rats (26), ewes (27), or monkeys (28) but to increase PRL release from bovine (29) and fish (30) pituitary cells.

In the present study, we used *in vivo* experimental approaches, double-label immunohistochemistry, neurochemical analysis, and primary cell culture to investigate the role of kisspeptin in the control of hypothalamic dopaminergic neurons and PRL secretion in adult rats.

Materials and Methods

Animals

For experiments 1, 2, and 3, female and male Wistar rats weighing 250–300 g were housed in groups of four per cage ($39 \times 32 \times 17$ cm) under conditions of controlled lighting (lights on 0600–1800 h) and temperature (22 ± 0.5 C), and experimental protocols were approved by the Ethics Committee on the Use of Experimental Animals of the University of São Paulo, Ribeirão Preto. For experiment 4, female Sprague Dawley rats weighing 250–300 g were grouped housed under conditions of controlled lighting (lights on from 0600–1800 h) and temperature (22 ± 1 C), and experimental protocols were approved by the University of Otago Animal Ethics Committee. In all experiments, food and water were provided *ad libitum*.

Experimental design

Experiment 1: effect of Kp-10 on PRL release and Fos-related antigen (FRA) expression in hypothalamic dopaminergic neurons

Rats were ovariectomized (OVX) and implanted with a guide cannula into the right lateral cerebral ventricle. Rats were treated 7–10 d after surgery with corn oil (OVX; 0.2 ml/rat, sc) or E2 (OVX+E2; E2 cypionate, Pfizer, São Paulo, Brazil; 10 μg/0.2 ml/rat, sc) daily for a consecutive 3 d, which yields physiological levels of plasma E2 (31), and experiments were conducted on the fourth day. The jugular vein was cannulated 1 d before the experiment for serial blood sampling. Between 0800 and 1200 h, blood samples were withdrawn 5 min before and 5, 10, 15, 30, and 60 min after intracerebroventricular (icv) injection of 0.3, 1, or 3 nmol Kp-10 or vehicle (Veh) in OVX (n = 6–7 per group) and OVX+E2 (n = 4–6 per group) rats for evaluation of plasma PRL levels by RIA. For comparison, the efficacy of Kp-10 treatment on plasma LH levels was also determined in OVX+E2 rats receiving 0.3 nmol Kp-10 or Veh (n = 6 per group). After the last sample, rats were anesthetized and transcardially perfused. The brains of rats injected with 3 nmol Kp-10 and Veh were immunohistochemically processed for FRA and tyrosine hydroxylase (TH) double labeling in the ARC and Pe.

Experiment 2: effect of Kp-10 on PRL release in male and cycling female rats

Vaginal smears were taken daily, and only rats showing 4-d regular cycles were used. A guide cannula was implanted into the right lateral cerebral ventricle of males and cycling females, and experiments were conducted 10–14 d later. Cycling rats were studied in the second regular cycle after surgery. The jugular vein was cannulated for serial blood sampling 1 d before the experiment. Between 0800 and 1200 h, blood samples were withdrawn 5 min before and 5, 10, 15, 30, and 60 min after icv injection of Kp-10 or Veh for plasma PRL measurement. Males received Kp-10 at 1 or 3 nmol (n = 5–7 per group). Females on proestrus (n = 6 per group) and diestrus (n = 6–7 per group) received Kp-10 at 3 nmol. Kp-10 doses were selected based on

results obtained in experiment 1. After the experiment, males and diestrous females were euthanized with sodium thiopental overdose. Proestrous females were euthanized on the next day to confirm the occurrence of ovulation. Only data from ovulating rats were used in the study.

Experiment 3: effect of Kp-10 on DA activity in the ME

Rats were OVX and implanted with a guide cannula into the right lateral cerebral ventricle. After surgery (7–10 d), rats were treated with E2 for 3 d, as described in experiment 1. Between 0800 and 1200 h on the fourth day, OVX+E2 rats were rapidly decapitated 10 min after icv injection of 3 nmol Kp-10 (n = 7) or Veh (n = 6). Trunk blood was collected for plasma PRL measurement. The brains were quickly removed, and the ME was dissected from the hypothalamus, frozen on dry ice, and stored at −70 C. As a control region the striatum (STR) was dissected using the punch technique (32). Concentrations of 3,4-dihydroxyphenylacetic acid (DOPAC) and DA were determined in the ME and STR microdissections by HPLC with electrochemical detection. DA turnover rate was estimated by the DOPAC-DA ratio.

Experiment 4: effect of Kp-10 on PRL release from anterior pituitary cell culture

To test whether Kp-10 would affect PRL secretion at the pituitary level, we measured PRL responses in isolated anterior pituitary cell cultures. Cycling rats were decapitated, and the anterior pituitary gland was collected into dispersion medium. Half of the cell cultures were treated with E2–17β (300 pg/ml) throughout the experiment, whereas the remainder received no steroid treatment. Pituitary cells were incubated with a culture medium containing 0, 10^{-9}, 10^{-8}, 10^{-7} or 10^{-6} M Kp-10 for 3 h (n = 6 for each treatment). The supernatant was harvested for PRL determination by RIA.

Intracerebroventricular microinjection

Under ketamine [80 mg/kg body weight (bw), ip] and xylazine (10 mg/kg bw, ip) anesthesia, rats were positioned in a stereotaxic instrument (David Kopf, Tujunga, CA) with the incisor bar at −3.3 mm. A 22-gauge stainless-steel guide cannula was implanted in the right cerebral lateral ventricle (coordinates: 1.0 mm posterior to the bregma, 1.6 mm lateral to the midline, and 3.2–3.7 mm below the outer surface of the skull). The displacement of the meniscus in a water manometer ensured correct positioning of the cannula in the lateral ventricle. The cannula, protected by a mandrel, was attached to the bone with stainless-steel screws and acrylic cement. After surgeries, rats were treated with pentabiotic (0.2 m/rat, im; Fort Dodge, Campinas, Brazil) and analgesic (Flunixin meglumine, 2.5 mg/kg, sc; Banamine, Schering-Plough, Rio de Janeiro, Brazil) and placed in individual cages. In experiment 1, ovariectomy immediately followed the stereotaxic surgery. The combined surgical procedures were performed over a period of 30–45 min. Kp-10 (metastin 45-54-amide, human; Phoenix Pharmaceuticals, Burlingame, CA) was dissolved in 0.01 M PBS, pH 7.33 (Veh). Injections were performed via a 30-gauge stainless-steel needle with an injection pump (KDS100; KD Scientific Inc., Holliston, MA) set to dispense 3 μl solution/min.

Jugular vein cannulation and blood samples

Under tribromoethanol anesthesia (250 mg/kg bw, ip), a SILASTIC brand cannula (Dow Corning Corp., Midland, MI) was inserted through the external jugular vein into the right atrium as previously described (33). After surgery, rats were treated with analgesic (Banamine, 2.5 mg/kg, sc). A length of polyethylene tubing (PE-50) filled with heparinized saline (0.9% NaCl, 30 IU heparin/ml) was connected to the jugular catheter. Blood samples of 500 μl were withdrawn into plastic heparinized syringes. After removal of each blood sample, an equal volume of sterile 0.9% NaCl was injected through the catheter. Plasma was separated by centrifugation at 1200 × *g* for 20 min at 4 C and stored at −20 C until hormonal assay.

Immunohistochemistry

Rats were deeply anesthetized with ketamine and xylazine and transcardially perfused with PBS, followed by 4% paraformaldehyde. Frontal sections of 20 μm were cut in four series between approximately −1.8 and −4.1 mm from bregma (34). Immunoperoxidase double labeling of FRA and TH was performed as previously described (31, 35). Briefly, sections were incubated with the anti-FRA rabbit antibody (K-25; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), at 1:2000 for 40 h, biotinylated antirabbit goat IgG (Vector Laboratories, Burlingame, CA) at 1:600 for 90 min, and avidin-biotin complex solution at 1:100 for 1 h (Elite ABC kit, Vector Laboratories). A solution of nickel sulfate (25 mg/ml), 3,3′-diaminobenzidine-HCl (DAB, 0.2 mg/ml) and 0.03% H_2O_2 (Ni-DAB) was used as the chromogen. Sections were then incubated with the anti-TH mouse antibody (anti-TH2; Sigma Chemical Co., St. Louis, MO) at 1:75,000 for 40 h, followed by biotinylated antimouse horse IgG (Vector Laboratories) at 1:600 for 1 h, and Elite ABC kit for 1 h. DAB (0.2 mg/ml) was used as chromogen. Omission of the primary antibodies resulted in no labeling, and antibody preadsorbtion with antigen peptide abolished FRA labeling (31). Brain sections were blindly analyzed for experimental groups under a light microscope with an image analysis system (Axiovision 3.1, Zeiss, Hallbergmoos, Germany). The number of TH-, FRA-, and FRA/TH-immunoreactive (ir) neurons was quantified bilaterally in the Pe (15 sections), rostral (R-ARC; three sections), middle (12 sections), and caudal (C-ARC; six sections) regions of the ARC (34). The middle portion was further divided into DM-ARC and VL-ARC subpopulations by a line forming a 50° angle with the horizontal plane in the midline (36).

For kisspeptin single labeling, ARC sections from OVX+E2 rats were incubated with antimouse Kp-10 antibody raised in rabbits (A.C. 564) (37), at 1:10,000 for 48 h, biotinylated antirabbit goat IgG (Vector Laboratories) at 1:600 for 2 h, and Elite ABC kit at 1:100 for 1 h. Ni-DAB solution was used as chromogen. As control for antigen specificity, kisspeptin antibody was preadsorbed overnight at 4 C with 1.5 μg/ml Kp-10 (metastin 45-54-amide, human; Phoenix Pharmaceuticals). Moreover, this antibody displays no cross-reaction with other related peptides (21, 37). For immunofluorescence double labeling of kisspeptin and TH, immunohistochemical procedures were conducted using streptavidin-fluorescence detection after biotin amplification for kisspeptin and fluorophore-tagged secondary antibody for TH, as previously described (38). Briefly, ARC sections from OVX+E2 rats were incubated with the anti-Kp-10 antibody, at 1:10,000 for 48 h, biotinylated antirabbit goat IgG (Vector Laboratories) at 1:5,000 for 1 h, Elite ABC kit at 1:880

for 30 min, biotinyltyramine (5 μl/ml) plus H_2O_2 (0.005%) for 20 min, and streptavidin-Texas Red (5 μl/ml) for 3 h. Sections were then incubated with the anti-TH mouse antibody (Millipore Corp., Bedford, MA) at 1:80,000 for 48 h, and Cy2-coupled antimouse donkey IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1:500 for 2 h. Staining was observed with a CARV Confocal Microscope (BioVision Technologies, Exton, PA) using filters for Texas Red or fluorescein. Images were captured through a Cook EX cooled digital camera (BioVision Technologies) and optically merged (0.2-μm optical sections).

HPLC-electrochemical detection

The ME was dissected with fine scissors using a dissecting microscope immediately after decapitation. The STR was dissected using the punch technique, as previously described (39). Using a 2-mm diameter needle, two punches of the STR were obtained bilaterally from a coronal section extending from approximately +0.12 to −0.88 mm from bregma (34). ME and STR dissections were homogenized in 150 or 300 μl, respectively, of 0.2 M perchloric acid, 0.1 mM EDTA, and 90 nM 3,4-dihydroxybenzylamine (internal standard; Aldrich, Milwaukee, WI). The homogenates were centrifuged for 20 min at 12,000 × *g*. Protein content was determined in the remaining pellet by the Bradford method (40). In the supernatant, DA and DOPAC concentrations were measured as previously described (41). Briefly, the separation was performed on a 250 × 4-mm C18 column (Purospher, 5 μm; Merck, Darmstadt, Germany), preceded by a 4 × 4-mm C18 guard column. The mobile phase consisted of 100 mM sodium dihydrogen phosphate; 10 mM sodium chloride; 0.1 mM EDTA; 0.19 mM sodium 1-octanesulfonic acid; and 13.5% methanol, pH 3.5. The pump flow rate was set at 0.45 ml/min. Chromatography data were plotted with Class-VP software (Shimadzu, Kyoto, Japan). All samples from each brain area were measured in the same analysis. The intraassay coefficient of variation was 0.6% for DA and 0.9% for DOPAC. DA levels were considered to reflect neurotransmitter in synaptic vesicles, whereas DOPAC levels reflected the amount of DA released in the sample (42). DOPAC-DA ratio was used as a measure of neurotransmitter turnover.

Anterior pituitary culture

Cycling rats were decapitated, and the anterior pituitary gland was collected into dispersion medium as previously described (43). The pituitaries were then cut in pieces of approximately 0.5 mm, centrifuged, and resuspended for 20 min at 37 C in dispersion medium containing 0.3% trypsin and 0.1% deoxyribonuclease. Pituitary fragments were again centrifuged and resuspended in dispersion medium containing 0.2% trypsin inhibitor and 0.1% deoxyribonuclease, triturated for several minutes, filtered through a 40-μm cell strainer and centrifuged. The cells were then resuspended in a culture medium (Medium 199; Invitrogen, Carlsbad, CA) supplemented with 0.2% $NaHCO_3$, 0.6% HEPES, 0.1% BSA, 100 U/ml each of penicillin and streptomycin, and 10% fetal calf serum at a concentration of 5×10^5 cells/ml. The pituitary cell suspension was aliquoted (500 μl) into 48-well tissue culture coated plates (BD Falcon, San Jose, CA) and maintained in a 5% CO_2-humidified incubator at 37 C. Half of the wells were treated with E2-17β (300 pg/ml) throughout the experiment, whereas the remainder received no steroid treatment. After 4 d, the cells were washed in new culture medium for 1 h immediately before treatment with culture medium containing 0, 10^{-9}, 10^{-8}, 10^{-7}, or 10^{-6} M Kp-10 (Calbiochem, La Jolla, CA) for 3 h (n = 6, for each treatment). The supernatant was harvested and stored at −20 C for later PRL RIA.

RIA

PRL and LH were assayed by double-antibody RIA with kits provided by the National Hormone and Peptide Program (Harbor-UCLA). The antiserum and reference preparation for PRL were antirat PRL-S9 and PRL-RP3, respectively. The antiserum and reference preparation for LH were antirat LH-S10 and LH-RP3, respectively. All samples of the same experiment were assayed in one RIA. The lower limits of detection were 0.7 ng/ml and 0.16 ng/ml for PRL and LH, respectively. The intraassay coefficients of variation were 3.9% and 4% for PRL and LH, respectively.

Statistical analysis

Data are presented as mean ± SEM. In experiments 1 and 2, PRL and immunohistochemical data were analyzed by two-way ANOVA. Differences in PRL levels among times within the same experimental group were determined by one-way ANOVA for repeated measures. Integrated PRL-secretory responses were expressed as the area under curve (AUC), determined by the trapezoid rule. Differences in the AUC were determined by one-way or two-way ANOVA. In experiment 3, DA and PRL data were analyzed by Student's *t* test. In experiment 4, PRL data from pituitary cell culture were analyzed by two-way and one-way ANOVA. In all analyses, ANOVA was followed by the Bonferroni *post hoc* test. $P < 0.05$ was considered statistically significant.

Results

Effect of Kp-10 on PRL release in OVX rats with or without E2 treatment

Figure 1 shows the effect of icv administration of Kp-10 on PRL release in OVX+E2 and OVX rats. As determined by two-way ANOVA, Kp-10 significantly altered PRL release in OVX+E2 rats ($P < 0.001$). Plasma PRL was higher at 5 and 10 min after injection of 3 nmol Kp-10 compared with Veh ($P < 0.05$). Compared with −5 min, plasma PRL was significantly increased 15 min after injection of 1 nmol, and at 5 and 10 min after 3 nmol Kp-10 ($P < 0.05$). PRL levels were not altered by 0.3 nmol Kp-10 (Fig. 1A). The AUC showed a dose-response effect of Kp-10 on integrated PRL secretory response in OVX+E2 rats, with significant effects at 1 and 3 nmol ($P < 0.05$; Fig. 1B). On the other hand, Kp-10 was ineffective in altering plasma PRL in OVX rats (Fig. 1, A and B). Basal PRL levels (−5 min) were higher in OVX+E2 than in OVX rats (9.7 ± 0.5 *vs.* 6.6 ± 0.6 ng/ml; $P < 0.001$). For comparison, LH levels in OVX+E2 rats receiving 0.3 nmol Kp-10 were significantly increased at 5–60 min after injection compared with Veh or −5 min ($P < 0.05$; Fig. 1C).

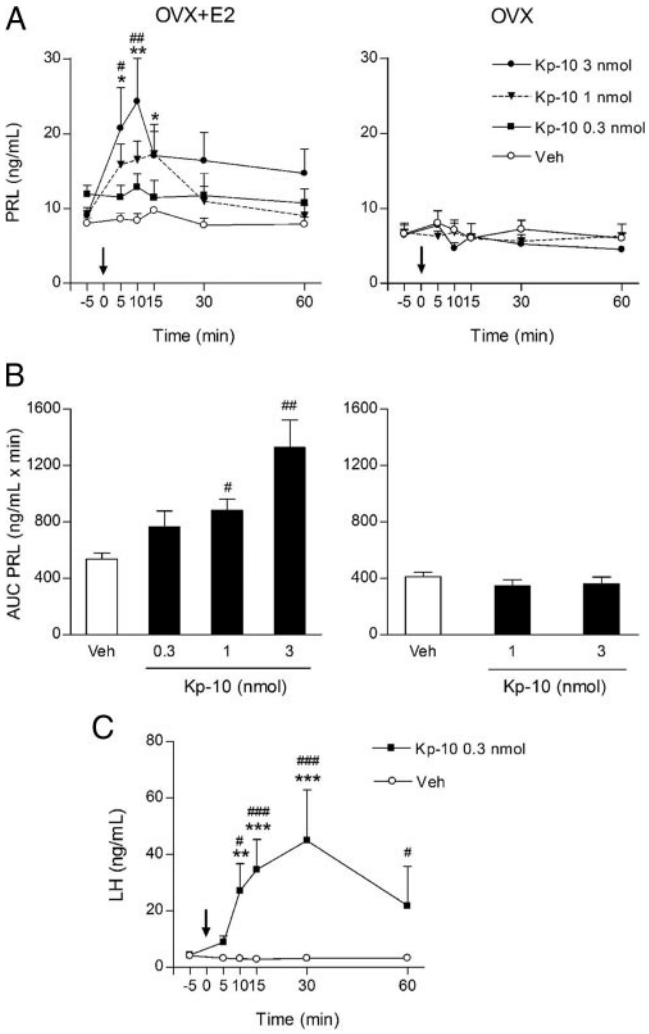

FIG. 1. Kp-10 increases PRL release in E2-treated OVX rats. Between 0800 and 1200 h, blood samples were withdrawn from the jugular vein catheter 5 min before and 5, 10, 15, 30, and 60 min after icv injection (*arrow*) of Kp-10 or Veh in OVX rats treated with E2 (OVX+E2; n = 4–6 per group) or oil (OVX, n = 6–7 per group). A, Plasma PRL levels in OVX+E2 rats receiving 0.3, 1, or 3 nmol Kp-10 or Veh, and in OVX rats receiving 1 or 3 nmol Kp-10 or Veh. Kp-10 (3 nmol): #, $P < 0.05$, ##, $P < 0.01$ compared with Veh ($F_{3,102} = 11.59$; $P < 0.001$); *, $P < 0.05$; **, $P < 0.01$ compared with −5 min ($F_{5,15} = 3.55$; $P < 0.05$). Kp-10 (1 nmol): *, $P < 0.05$ compared with −5 min ($F_{5,25} = 3.62$; $P < 0.05$). B, AUC of plasma PRL levels over the study period. #, $P < 0.05$; ##, $P < 0.01$ compared with Veh ($F_{3,15} = 6.81$; $P < 0.01$). C, Plasma LH levels in OVX+E2 rats receiving 0.3 nmol Kp-10 or Veh (n = 6 per group). #, $P < 0.05$; ###, $P < 0.001$ compared with Veh ($F_{1,60} = 77.66$; $P < 0.001$); **, $P < 0.01$; ***, $P < 0.001$ compared with −5 min ($F_{5,25} = 8.45$, $P < 0.001$). Data shown as mean ± SEM.

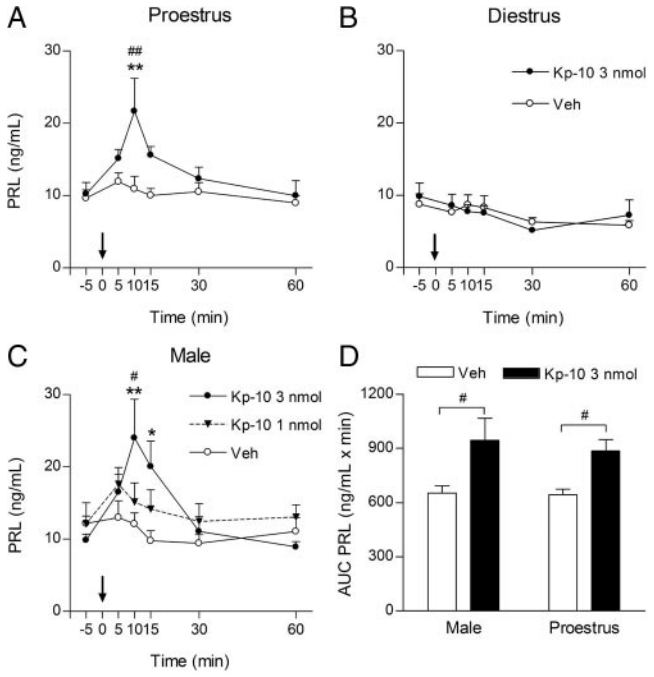

FIG. 2. Kp-10 increases PRL release in male and cycling female rats. Between 0800 and 1200 h, blood samples were withdrawn from the jugular vein catheter 5 min before and 5, 10, 15, 30, and 60 min after icv injection (*arrow*) of Kp-10 or Veh. A and B, Plasma PRL levels in proestrous (n = 6 per group) and diestrous (n = 6–7 per group) females receiving 3 nmol Kp-10 or Veh. ##, $P < 0.01$ compared with Veh ($F_{1,60} = 12.46$, $P < 0.001$); **, $P < 0.01$ compared with −5 min ($F_{5,25} = 4.56$; $P < 0.01$). C, Plasma PRL levels in male rats (n = 5–7 per group) receiving 1 or 3 nmol Kp-10 or Veh. #, $P < 0.05$ compared with Veh ($F_{2,89} = 3.98$; $P < 0.05$); *, $P < 0.05$; **, $P < 0.01$ compared with −5 min ($F_{5,25} = 6.04$; $P < 0.001$). D, AUC of plasma PRL levels over the study period. #, $P < 0.05$ compared with Veh ($F_{1,17} = 14.60$; $P < 0.01$). Data shown as mean ± SEM.

Effect of Kp-10 on PRL release in male and cycling female rats

Figure 2 shows the effect of icv injection of Kp-10 on PRL release in males and in females during proestrus or diestrus. Rats on proestrus exhibited an ovulation rate of 11.5 ± 1.9 ova. As determined by two-way ANOVA, Kp-10 significantly altered PRL release in proestrous rats ($P < 0.001$), with higher plasma levels 10 min after injection of 3 nmol Kp-10 compared with Veh or −5 min ($P < 0.01$). In contrast, 3 nmol Kp-10 was unable to alter plasma PRL in diestrous rats (Fig. 2B). Kp-10 significantly altered PRL release in male rats, with higher levels 10 min after injection of 3 nmol Kp-10 compared with Veh ($P < 0.05$). Compared with −5 min, plasma PRL was increased at 10 and 15 min by 3 nmol ($P < 0.05$) but not altered by 1 nmol Kp-10 (Fig. 2C). As determined by the AUC, PRL response to 3 nmol Kp-10 did not differ between males and proestrous females (Fig. 2D).

Effect of Kp-10 on FRA expression in hypothalamic dopaminergic neurons

Figure 3 shows representative photomicrographs of FRA and TH double labeling in the DM-ARC (Fig. 3, A and B) and Pe (Fig. 3, C and D). Figure 4 shows the effect of icv 3 nmol Kp-10 on FRA expression in TH-ir neurons of ARC and Pe in OVX+E2 and OVX rats. OVX+E2 rats displayed a higher percentage of TH-ir neurons expressing FRA in the R-ARC, DM-ARC, VL-ARC, and Pe than OVX rats ($P < 0.05$). Kp-10, in turn, significantly decreased FRA expression in TH-ir neurons of R-ARC, DM-

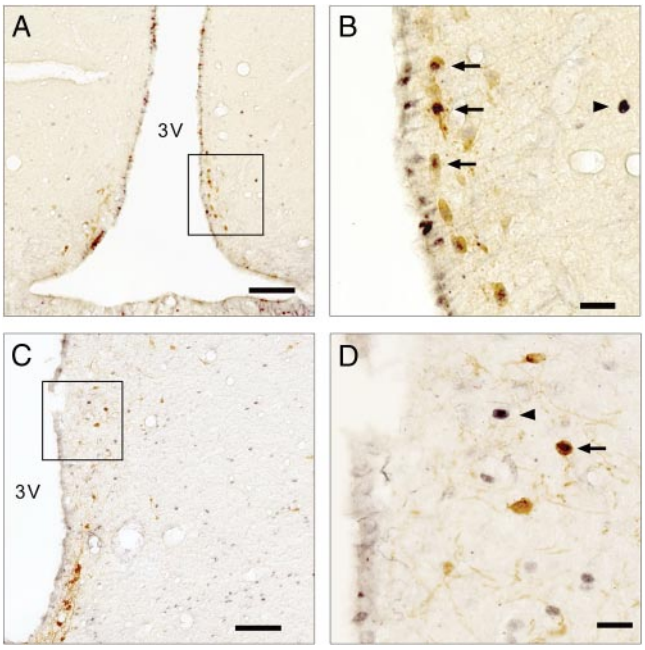

FIG. 3. Representative photomicrographs of FRA/TH double labeling in coronal sections of DM-ARC (A and B) and Pe (C and D) nuclei. B and D, Higher magnification of the squared areas in DM-ARC and Pe nuclei, respectively. *Arrows* indicate double-labeled neurons (TH staining is cytoplasmic, and FRA is nuclear). *Arrowheads* indicate single-labeled FRA-ir neurons. 3V, Third ventricle. *Scale bars*, 100 μm (A and C) and 20 μm (B and D).

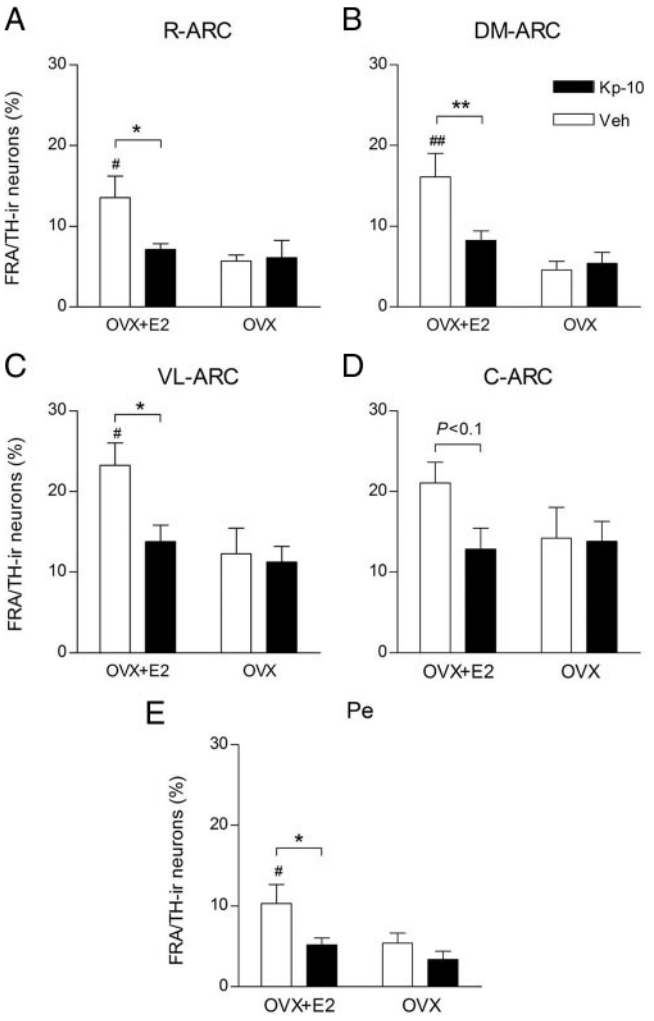

FIG. 4. Kp-10 decreases FRA expression in TH-ir neurons of ARC and Pe nuclei. Mean ± SEM percentage of TH-ir neurons expressing FRA in the rostral (panel A, R-ARC), DM (panel B, DM-ARC), VL (panel C, VL-ARC), caudal (panel D, C-ARC) ARC, and Pe (panel E) nuclei of OVX rats treated with E2 (OVX+E2, n = 5–6 per group) or oil (OVX, n = 6 per group) perfused approximately 60 min after icv injection of 3 nmol Kp-10 or Veh\. #, $P < 0.05$; ##, $P < 0.01$ compared with OVX in the R-ARC ($F_{1,17} = 11.58$; $P < 0.01$), DM-ARC ($F_{1,19} = 18.52$, $P < 0.001$); VL-ARC ($F_{1,18} = 8.27$, $P < 0.05$); and Pe ($F_{1,19} = 5.80$, $P < 0.05$). *, $P < 0.05$; **, $P < 0.01$ Kp-10 *vs.* Veh in the R-ARC ($F_{1,17} = 6.14$, $P < 0.05$); DM-ARC ($F_{1,19} = 4.52$, $P < 0.05$); VL-ARC ($F_{1,18} = 5.25$, $P < 0.05$); and Pe ($F_{1,19} = 6.55$, $P < 0.05$).

ARC, VL-ARC, and Pe in OVX+E2 ($P < 0.05$), whereas it had no effect in OVX rats. In OVX+E2 C-ARC, there was a trend toward decrease in FRA/TH-ir neurons after Kp-10 injection ($P < 0.1$; Fig. 4D). In addition, the number of single labeled TH-ir and FRA-ir neurons was not altered by Kp-10 (Table 1).

Effect of Kp-10 on DA activity in the ME

Figure 5 shows the effect of icv 3 nmol Kp-10 on DA and DOPAC concentrations and DOPAC-DA ratio in the ME and STR of OVX+E2 rats. Kp-10 significantly decreased DOPAC levels and DOPAC-DA ratio in the ME ($P < 0.05$; Fig. 5A), and this effect was associated with an increase in plasma PRL levels ($P < 0.01$; Fig. 5C). In contrast, DOPAC and DOPAC-DA ratio in the STR were not modified by Kp-10 (Fig. 5B).

Kisspeptin immunoreactivity in the ARC nucleus

Figure 6 shows representative photomicrographs of kisspeptin immunoreactivity in the ARC of the OVX+E2 rat. A high density of kisspeptin-ir fibers and evenly distributed neurons were found throughout the rostro-caudal extension of the ARC (Fig. 6, A and B), in agreement with previous reports in OVX+E2 rats (44). Confirming the selectivity of the Kp-10 antibody (A. C. 564), kisspeptin staining was greatly diminished by antibody preadsorbtion with 1.5 μg/ml human Kp-10 (Fig. 6C). However, some faintly labeled neurons were still observed, consistent with previous reports (21, 37). Confocal microscopy images revealed kisspeptin-ir fibers in close apposition to TH-ir perikarya, displaying points of contact (Fig. 6D). In addition, TH-ir and kisspeptin-ir cell bodies were not found to be colocalized in the ARC.

Effect of Kp-10 on PRL release from anterior pituitary cell culture

Figure 7 shows PRL release from primary cultures of pituitary cells in response to Kp-10 incubation in the presence or absence of E2. As determined by two-way ANOVA, there was a main effect for E2 ($P < 0.05$) but

TABLE 1. Number of TH- and FRA-ir neurons per section in the ARC and Pe nuclei

		OVX+E2		OVX	
Brain area	**Neurons**	**Veh**	**Kp-10**	**Veh**	**Kp-10**
R-ARC	TH-ir	57.1 ± 7.3	54.8 ± 1.8	63.3 ± 2.6	55.1 ± 3.2
	FRA-ir	5.0 ± 1.7	3.5 ± 0.9	5.2 ± 1.2	4.4 ± 1.4
DM-ARC	TH-ir	28.8 ± 5.9	29.4 ± 2.0	31.4 ± 1.8	29.4 ± 2.3
	FRA-ir	1.5 ± 0.4	1.5 ± 0.3	2.4 ± 0.9	2.2 ± 0.4
VL-ARC	TH-ir	6.9 ± 1.3	6.0 ± 0.6	9.8 ± 0.8[a]	9.2 ± 1.1[a]
	FRA-ir	12.4 ± 3.0	8.0 ± 0.7	12.2 ± 2.1	11.7 ± 2.5
C-ARC	TH-ir	8.5 ± 0.6	10.3 ± 1.5	9.8 ± 0.6	10.0 ± 0.8
	FRA-ir	17.3 ± 3.6	14.6 ± 1.4	18.8 ± 2.5	15.4 ± 2.1
Pe	TH-ir	6.7 ± 1.3	6.0 ± 0.4	7.0 ± 0.7	5.9 ± 0.4
	FRA-ir	5.5 ± 1.6	3.6 ± 1.3	4.4 ± 0.4	4.0 ± 1.0

OVX rats treated with E2 (OVX+E2, n = 5–6, per group) or oil (OVX, n = 6, per group) were perfused approximately 60 min after icv injection of 3 nmol Kp-10 or Veh. Mean ± SEM number of TH-ir or FRA-ir neurons per section in the R-ARC, DM-ARC, VL-ARC, C-ARC, and Pe.

[a] $P < 0.05$ compared with OVX+E2 for TH-ir neurons ($F_{1,18} = 9.72$; $P < 0.01$). Number of FRA-ir neurons did not differ significantly among groups.

no effect for Kp-10 ($P = 0.28$) or interaction between these two factors ($P = 0.90$). Kp-10 did not alter PRL release at any of the doses evaluated either in the presence ($P = 0.14$) or absence ($P = 0.76$) of E2. The experiment was repeated on a separate occasion with essentially identical results.

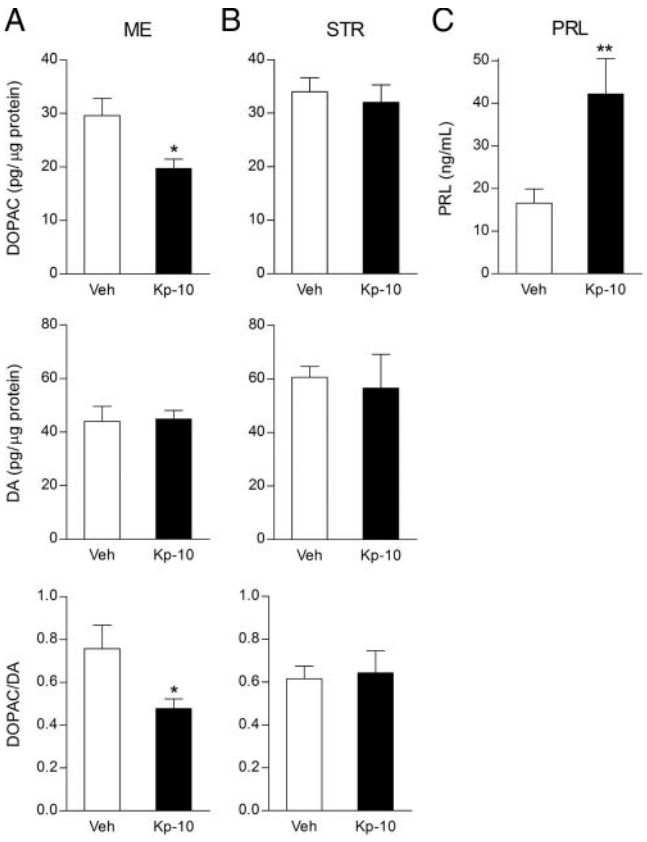

FIG. 5. Kp-10 decreases DA activity in the ME of E2-treated OVX (OVX+E2) rats. Between 0800 and 1200 h, OVX+E2 rats were decapitated 10 min after icv injection of 3 nmol Kp-10 (n = 7) or Veh (n = 6). DOPAC, DA, and DOPAC-DA ratio were determined in the ME (A) and striatum (panel B, STR). Plasma PRL levels were evaluated (C). Data shown as mean ± SEM; *, $P < 0.05$; **, $P < 0.01$ compared with Veh for ME DOPAC ($t_{11} = 2.83$; $P < 0.05$) and DOPAC/DA ($t_{11} = 2.53$; $P < 0.05$), and plasma PRL ($t_{10} = 3.33$; $P < 0.01$).

Discussion

Taken together, our data provide compelling evidence supporting a role for ARC kisspeptin neurons in the regulation of hypothalamic DA and thereby PRL secretion. Kp-10 icv increased PRL release in a dose-dependent manner. This stimulatory effect was found in adult female and male rats, being E2 dependent in females. The Kp-10 induced increase in PRL release was associated with a decrease in FRA expression in TH-ir neurons of the ARC and Pe and in dopaminergic activity in the ME. Kisspeptin fibers were observed in close apposition to and in contact with ARC dopaminergic neurons. In contrast, Kp-10 did not alter PRL release from anterior pituitary cell cultures, reinforcing the notion that the PRL-releasing effects of Kp-10 are primarily due to actions in the brain.

PRL secretion is known to be tonically inhibited by DA (3). FRA expression has proved to be useful in determining changes in the activity of ARC dopaminergic neurons (45). The higher percentage of ARC and Pe TH-ir neurons expressing FRA in OVX+E2 compared with OVX is consistent with previous studies (46) and likely reflects the higher activity of these neurons due to the short-loop feedback of PRL, stimulated by E2 treatment (47). The decreased FRA expression in TH-ir neurons of ARC and Pe after Kp-10 injection in OVX+E2 rats suggests reduction in the activity of TIDA, tuberohypophyseal dopaminergic, and periventricular hypophyseal dopaminergic neurons, implicated in tonic inhibition of PRL release. This response is probably E2 dependent because Kp-10 had no effect in OVX rats. Moreover, Kp-10 decreased FRA expression in OVX+E2 rats to the levels found in OVX,

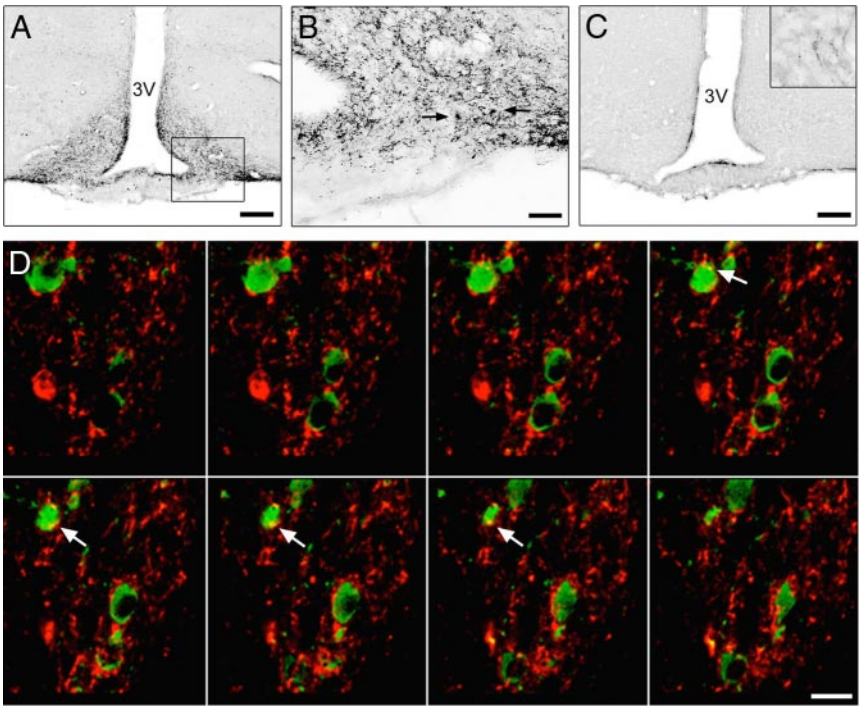

FIG. 6. Kisspeptin and TH immunoreactivity in the rat ARC nucleus. OVX rats treated with E2 were perfused between 0800 and 1200 h. A, Representative photomicrograph of kisspeptin immunoreactivity in the ARC. B, Higher magnification of the squared area in panel A, showing kisspeptin-ir neurons (*arrows*) and fibers in the ARC. C, Marked reduction of kisspeptin staining after antibody preadsorbtion with 1.5 μg/ml human Kp-10. *Inset*, In higher magnification, a faintly labeled neuron remaining after antibody preadsorbtion (see *Results*). D, Series of confocal fluorescent images showing kisspeptin (*red*) and TH (*green*) double labeling in the ARC. Kisspeptin-ir fibers are in close apposition to TH-ir perikarya. *Arrows* indicate points of contact (0.2-μm optical sections). 3V, Third ventricle. *Scale bars*, 200 μm (A and C), 20 μm (B), and 10 μm (D).

suggesting that E2 facilitatory effect involves increase in the basal activity of dopaminergic neurons. DOPAC is considered to be a reliable index of TIDA neuron activity (42). Thus, data of FRA expression in TH-ir neurons were confirmed by the decrease in DOPAC concentration and DOPAC-DA ratio in the ME, associated with the increase in PRL release, elicited by Kp-10 in OVX+E2 rats. Taken together, these data suggest that the PRL-releasing effect of kisspeptin is mediated through inhibition of dopaminergic neurons and therefore DA release in the ME. Interestingly, a similar pattern of interaction seems to occur between kisspeptin and γ-aminobutyric acid. Kp-10 has been reported to inhibit γ-aminobutyric acid release in the preoptic area of middle-aged rats, restoring the steroid-induced LH surge (48).

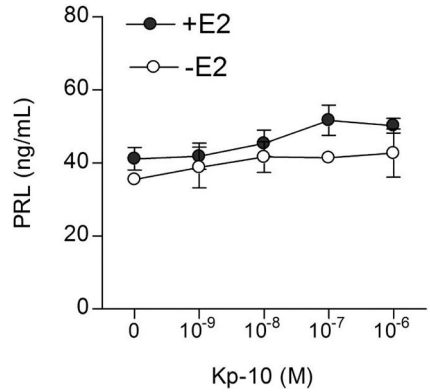

FIG. 7. Effect of Kp-10 on PRL release from anterior pituitary cells. Half of the cell cultures were treated with E2–17β (300 pg/ml) throughout the experiment (+E2), whereas the remainder received no steroid treatment (−E2). Data shown as mean ± SEM of PRL concentrations in culture media of anterior pituitary cells (5×10^5 cells/ml) incubated with 0, 10^{-9}, 10^{-8}, 10^{-7}, or 10^{-6} M Kp-10 for 3 h (n = 6 for each treatment). There was significant main effect for E2 ($F_{1,47} = 5.43$, $P < 0.05$) but no effect for Kp-10 ($F_{4,17} = 1.31$; $P = 0.28$) or interaction between these two factors ($F_{4,17} = 0.26$; $P = 0.90$).

Two lines of evidence suggest selectivity of kisspeptin action upon hypothalamic dopaminergic neurons. One is the absence of effect of Kp-10 on FRA expression in non-TH-ir neurons of ARC and Pe, and the other is that Kp-10 did not alter the activity of dopaminergic terminals in the STR. Thus, kisspeptin actions in the ARC seem to be directed mainly toward DA neurons. ARC kisspeptin neurons have been recently suggested to be interconnected via recurrent collaterals (22), but it remains to be determined whether kisspeptin is able to regulate its own release. Moreover, the population of DA neurons in the nigrostriatal system, which is under estrogen-regulatory effects but not involved in PRL secretion (49), is probably not regulated by kisspeptin.

Double-label immunofluorescence combined with confocal microscopy revealed that kisspeptin fibers are in close apposition to, and display contact with, dopaminergic neurons in the ARC. In addition, kisspeptin and TH-ir cell bodies were not found to be colocalized in the ARC, in contrast with kisspeptin population in the AVPV that may coexpress TH (50). Essentially all kisspeptin neurons have been reported to coexpress dynorphin in the sheep and mouse ARC (21, 22), and we have found the same pattern of coexpression in the rat ARC (G.E.H., unpublished data). Moreover, dynorphin terminals are known to display synaptic contacts with ARC dopaminergic neurons (23). It is therefore very likely that kisspeptin/dynorphin fibers make synaptic contact with dopaminergic neurons in the ARC, constituting the neuronal pathway for PRL regulation.

E2 is known to exert major stimulatory influence on PRL homeostasis. E2 stimulates PRL synthesis and release

by the lactotrophs (51, 52) and reduces lactotrophs sensitivity to DA (53). Accordingly, in the present study E2 promoted higher basal release of PRL in OVX+E2, compared with OVX rats, and in anterior pituitary cell cultures. Moreover, Kp-10 elicited PRL release in an E2-dependent manner in females, because its stimulatory effects were found in OVX+E2 and proestrous rats but not in OVX or diestrous rats.

The expression of *KiSS1* mRNA in the ARC is inhibited by gonadal steroids in both male and female rodents (16, 17); and in cycling rats, *KiSS1* expression is higher on diestrus compared with proestrus (18). We hypothesize the priming effect of E2 in the observed PRL response may involve the sensitization of ARC dopaminergic neurons by altering KiSS1R expression or activity. For that, E2 could either act directly on dopaminergic neurons, or indirectly through the regulation of ARC *KiSS1* expression. In the second possibility, the higher *KiSS1* expression in states of low circulating E2 might desensitize dopaminergic neurons to the inhibitory effect of kisspeptin, and therefore preclude PRL response to Kp-10 treatment. It remains to be determined whether ARC dopaminergic neurons contain KiSS1R and whether its expression or activity is regulated by E2. KiSS1R is expressed in the ARC (54), but only a slight if any effect of E2 on KiSS1R gene expression has been found in this brain area (55). Alternatively, the insensitivity of dopaminergic neurons to Kp-10 in OVX rats might occur at the intracellular signaling level, as is the case with their insensitivity to PRL feedback during late pregnancy and lactation (56). In addition, in rodents the population of ARC kisspeptin neurons is sexually undifferentiated, and testosterone inhibition of *KiSS1* mRNA expression in males is mediated, at least in part, by its aromatization to E2 (17, 50). These developmental and functional similarities between sexes raise the possibility that in males the PRL-releasing effect of kisspeptin may also depend on the levels of testosterone, or E2 after aromatization. This is an interesting possibility that needs further studies to be confirmed.

Even at high doses Kp-10 failed to modify PRL release from anterior pituitary cell regardless of E2 treatment. This is in contrast to previous reports that Kp-10 increases PRL release from bovine (29) and fish (30) pituitary cells. Indeed, KiSS1R is expressed in ovine lactotrophs (57). However, high doses of Kp-10 (10^{-5} to 10^{-6} M) were required to release PRL in bovine pituitaries. Thus, one may suppose that PRL could have been stimulated *in vitro* had we used higher doses of Kp-10. Conversely, lower doses of Kp-10 were required in goldfish pituitaries (30), suggesting pituitary cells are probably more responsive to kisspeptin in fish than in mammals. In our experiment, E2 was added to the cells in culture. Although this model does not replicate systemic effects of E2 in OVX+E2 or proestrous rats, it provided evidence that the E2-facilitatory effect on PRL response is not due to actions at the pituitary level. Moreover, Kp-10 up to the micromolar range did not stimulate PRL release from pituitary cells, which reinforces the major importance of central actions of kisspeptin for PRL regulation.

As a matter of comparison, robust LH release was achieved with a lower dose of Kp-10 than that required for PRL, which is consistent with the established high sensitivity of GnRH/LH to kisspeptin (11, 12). Thus, PRL is less responsive than LH to the stimulatory effect of kisspeptin, and contradictions between our findings and previous studies may be explained by the dose and mode of administration used. Kp-10 injected iv was not found to alter PRL release in ewes, goats, or monkeys (27, 28, 58). It seems therefore that icv administration is more effective than iv to decrease activity of dopaminergic neurons and increase PRL release. Our data showed that 1 nmol Kp-10 increases PRL release in OVX+E2 but not in male rats, suggesting that males have a higher threshold for PRL response than females. This is in accordance with the previous report that 1 nmol Kp-10 icv did not alter PRL in adult male rats (26).

Distinct neuroendocrine systems are implicated in PRL and LH regulation. It seems reasonable, therefore, that inhibition of dopaminergic neurons probably involves cellular and integrative mechanisms other than those for stimulation of GnRH neurons. This may explain the higher dose of Kp-10 needed as well as the distinct pattern of PRL response compared with LH. Nevertheless, the fact that PRL was stimulated by Kp-10 in the low nanomol range suggests that kisspeptin is implicated in the physiological secretion of PRL. We hypothesize that kisspeptin may play a role in PRL regulation in conditions such as the afternoon of proestrus and pregnancy (59, 60), which are characterized by sustained high levels of kisspeptin (55, 61–63).

Our morphological and functional data support kisspeptin involvement in the regulation of hypothalamic dopamine and PRL. These findings raise intriguing questions about the physiological relevance of this new regulatory pathway and the impact of kisspeptin and PRL interaction on the regulation of HPG axis.

Acknowledgments

We thank Maicon R. Silva and Ruither O.G. Carolino (Universidade de São Paulo, Ribeirão Preto, Brazil) for technical support. We also thank to Dr. Alain Caraty (Université de Tours, Nouzilly, France) for the generous gift of antikisspeptin antibody and Dr. Tony M. Plant (University of Pittsburgh, Pittsburgh, PA) for helpful comments on the manuscript.

Address all correspondence and requests for reprints to: Raphael E. Szawka, Departamento de Fisiologia e Biofisica, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Avenida Antônio Carlos 6627, 31270-910 Belo Horizonte, Minas Gerais, Brazil. E-mail: reszawka@icb.ufmg.br (current address).

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo and Conselho Nacional de Desenvolvimento Cientifico e Tecnológico.

Disclosure Summary: The authors have nothing to disclose.

References

1. **Riddle O, Bates RW, Dykshorn SW** 1933 The preparation, identification and assay of prolactin: a hormone of the anterior pituitary. Am J Physiol 105:191–216
2. **Bole-Feysot C, Goffin V, Edery M, Binart N, Kelly PA** 1998 Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. Endocr Rev 19:225–268
3. **Freeman ME, Kanyicska B, Lerant A, Nagy G** 2000 Prolactin: structure, function, and regulation of secretion. Physiol Rev 80:1523–1631
4. **Kawano H, Daikoku S** 1987 Functional topography of the rat hypothalamic dopamine neuron systems: retrograde tracing and immunohistochemical study. J Comp Neurol 265:242–253
5. **Holzbauer M, Racké K** 1985 The dopaminergic innervation of the intermediate lobe and of the neural lobe of the pituitary gland. Med Biol 63:97–116
6. **Goudreau JL, Falls WM, Lookingland KJ, Moore KE** 1995 Periventricular-hypophysial dopaminergic neurons innervate the intermediate but not the neural lobe of the rat pituitary gland. Neuroendocrinology 62:147–154
7. **Grattan DR, Kokay IC** 2008 Prolactin: a pleiotropic neuroendocrine hormone. J Neuroendocrinol 20:752–763
8. **Ohtaki T, Shintani Y, Honda S, Matsumoto H, Hori A, Kanehashi K, Terao Y, Kumano S, Takatsu Y, Masuda Y, Ishibashi Y, Watanabe T, Asada M, Yamada T, Suenaga M, Kitada C, Usuki S, Kurokawa T, Onda H, Nishimura O, Fujino M** 2001 Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. Nature 411:613–617
9. **Gottsch ML, Clifton DK, Steiner RA** 2009 From KISS1 to kisspeptins: an historical perspective and suggested nomenclature. Peptides 30:4–9
10. **Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno Jr JS, Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG, Zahn D, Dixon J, Kaiser UB, Slaugenhaupt SA, Gusella JF, O'Rahilly S, Carlton MB, Crowley Jr WF, Aparicio SA, Colledge WH** 2003 The GPR54 gene as a regulator of puberty. N Engl J Med 349:1614–1627
11. **Gottsch ML, Cunningham MJ, Smith JT, Popa SM, Acohido BV, Crowley WF, Seminara S, Clifton DK, Steiner RA** 2004 A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. Endocrinology 145:4073–4077
12. **Navarro VM, Castellano JM, Fernández-Fernández R, Tovar S, Roa J, Mayen A, Nogueiras R, Vazquez MJ, Barreiro ML, Magni P, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M** 2005 Characterization of the potent luteinizing hormone-releasing activity of KiSS-1 peptide, the natural ligand of GPR54. Endocrinology 146:156–163
13. **Shahab M, Mastronardi C, Seminara SB, Crowley WF, Ojeda SR, Plant TM** 2005 Increased hypothalamic GPR54 signaling: a potential mechanism for initiation of puberty in primates. Proc Natl Acad Sci USA 102:2129–2134
14. **Irwig MS, Fraley GS, Smith JT, Acohido BV, Popa SM, Cunningham MJ, Gottsch ML, Clifton DK, Steiner RA** 2004 Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat. Neuroendocrinology 80:264–272
15. **Han SK, Gottsch ML, Lee KJ, Popa SM, Smith JT, Jakawich SK, Clifton DK, Steiner RA, Herbison AE** 2005 Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty. J Neurosci 25:11349–11356
16. **Smith JT, Cunningham MJ, Rissman EF, Clifton DK, Steiner RA** 2005 Regulation of Kiss1 gene expression in the brain of the female mouse. Endocrinology 146:3686–3692
17. **Smith JT, Dungan HM, Stoll EA, Gottsch ML, Braun RE, Eacker SM, Clifton DK, Steiner RA** 2005 Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse. Endocrinology 146:2976–2984
18. **Smith JT, Popa SM, Clifton DK, Hoffman GE, Steiner RA** 2006 Kiss1 neurons in the forebrain as central processors for generating the preovulatory luteinizing hormone surge. J Neurosci 26:6687–6694
19. **Roa J, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M** 2008 New frontiers in kisspeptin/GPR54 physiology as fundamental gatekeepers of reproductive function. Front Neuroendocrinol 29:48–69
20. **Yamada S, Uenoyama Y, Kinoshita M, Iwata K, Takase K, Matsui H, Adachi S, Inoue K, Maeda KI, Tsukamura H** 2007 Inhibition of metastin (kisspeptin-54)-GPR54 signaling in the arcuate nucleus-median eminence region during lactation in rats. Endocrinology 148:2226–2232
21. **Goodman RL, Lehman MN, Smith JT, Coolen LM, de Oliveira CV, Jafarzadehshirazi MR, Pereira A, Iqbal J, Caraty A, Ciofi P, Clarke IJ** 2007 Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin A and neurokinin B. Endocrinology 148:5752–5760
22. **Navarro VM, Gottsch ML, Chavkin C, Okamura H, Clifton DK, Steiner RA** 2009 Regulation of gonadotropin-releasing hormone secretion by kisspeptin/dynorphin/neurokinin B neurons in the arcuate nucleus of the mouse. J Neurosci 29:11859–11866
23. **Fitzsimmons MD, Olschowka JA, Wiegand SJ, Hoffman GE** 1992 Interaction of opioid peptide-containing terminals with dopaminergic perikarya in the rat hypothalamus. Brain Res 581:10–18
24. **Arbogast LA, Voogt JL** 1998 Endogenous opioid peptides contribute to suckling-induced prolactin release by suppressing tyrosine hydroxylase activity and messenger ribonucleic acid levels in tuberoinfundibular dopaminergic neurons. Endocrinology 139:2857–2862
25. **Andrews ZB, Grattan DR** 2003 Opioid receptor subtypes involved in the regulation of prolactin secretion during pregnancy and lactation. J Neuroendocrinol 15:227–236
26. **Navarro VM, Castellano JM, Fernández-Fernández R, Barreiro ML, Roa J, Sanchez-Criado JE, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M** 2004 Developmental and hormonally regulated messenger ribonucleic acid expression of KiSS-1 and its putative receptor, GPR54, in rat hypothalamus and potent luteinizing hormone-releasing activity of KiSS-1 peptide. Endocrinology 145:4565–4574
27. **Smith JT, Saleh SN, Clarke IJ** 2009 Seasonal and cyclical change in the luteinizing hormone response to kisspeptin in the ewe. Neuroendocrinology 90:283–291
28. **Ramaswamy S, Gibbs RB, Plant TM** 2009 Studies of the localisation of kisspeptin within the pituitary of the rhesus monkey (*Macaca mulatta*) and the effect of kisspeptin on the release of non-gonadotropic pituitary hormones. J Neuroendocrinol 21:795–804
29. **Kadokawa H, Suzuki S, Hashizume T** 2008 Kisspeptin-10 stimulates the secretion of growth hormone and prolactin directly from cultured bovine anterior pituitary cells. Anim Reprod Sci 105:404–408
30. **Yang B, Jiang Q, Chan T, Ko WK, Wong AO** 2010 Goldfish kisspeptin: molecular cloning, tissue distribution of transcript expression, and stimulatory effects on prolactin, growth hormone and luteinizing hormone secretion and gene expression via direct actions at the pituitary level. Gen Comp Endocrinol 165:60–71
31. **Szawka RE, Rodovalho GV, Monteiro PM, Carrer HF, Anselmo-**

Franci JA 2009 Ovarian-steroid modulation of locus coeruleus activity in female rats: involvement in luteinising hormone regulation. J Neuroendocrinol 21:629–639
32. **Palkovits M** 1973 Isolated removal of hypothalamic or other brain nuclei of the rat. Brain Res 59:449–450
33. **Poletini MO, Szawka RE, Freitas Marcon RM, Veiga MD, Franci CR, Anselmo-Franci JA** 2003 A method to study preovulatory surges of gonadotropins. Brain Res Brain Res Protoc 12:41–48
34. **Paxinos G, Watson C** 2007 The rat brain in stereotaxic coordinates. 6th ed. San Diego: Elsevier Academic Press
35. **Bernuci MP, Szawka RE, Helena CV, Leite CM, Lara HE, Anselmo-Franci JA** 2008 Locus coeruleus mediates cold stress-induced polycystic ovary in rats. Endocrinology 149:2907–2916
36. **Leite CM, Szawka RE, Anselmo-Franci JA** 2008 α-Oestrogen and progestin receptor expression in the hypothalamus and preoptic area dopaminergic neurones during oestrous in cycling rats. J Neuroendocrinol 20:110–119
37. **Franceschini I, Lomet D, Cateau M, Delsol G, Tillet Y, Caraty A** 2006 Kisspeptin immunoreactive cells of the ovine preoptic area and arcuate nucleus co-express estrogen receptor α. Neurosci Lett 401: 225–230
38. **Guidetti P, Hoffman GE, Melendez-Ferro M, Albuquerque EX, Schwarcz R** 2007 Astrocytic localization of kynurenine aminotransferase II in the rat brain visualized by immunocytochemistry. Glia 55:78–92
39. **Szawka RE, Rodovalho GV, Helena CV, Franci CR, Anselmo-Franci JA** 2007 Prolactin secretory surge during estrus coincides with increased dopamine activity in the hypothalamus and preoptic area and is not altered by ovariectomy on proestrus. Brain Res Bull 73:127–134
40. **Bradford MM** 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
41. **Lima FB, Szawka RE, Anselmo-Franci JA, Franci CR** 2007 Pargyline effect on luteinizing hormone secretion throughout the rat estrous cycle: correlation with serotonin, catecholamines and nitric oxide in the medial preoptic area. Brain Res 1142:37–45
42. **Lookingland KJ, Jarry HD, Moore KE** 1987 The metabolism of dopamine in the median eminence reflects the activity of tuberoinfundibular neurons. Brain Res 419:303–310
43. **Anderson GM, Relf HL, Rizwan MZ, Evans JJ** 2009 Central and peripheral effects of RFamide-related peptide-3 on luteinizing hormone and prolactin secretion in rats. Endocrinology 150:1834–1840
44. **Adachi S, Yamada S, Takatsu Y, Matsui H, Kinoshita M, Takase K, Sugiura H, Ohtaki T, Matsumoto H, Uenoyama Y, Tsukamura H, Inoue K, Maeda K** 2007 Involvement of anteroventral periventricular metastin/kisspeptin neurons in estrogen positive feedback action on luteinizing hormone release in female rats. J Reprod Dev 53: 367–378
45. **Hoffman GE, Le WW, Abbud R, Lee WS, Smith MS** 1994 Use of Fos-related antigens (FRAs) as markers of neuronal activity: FRA changes in dopamine neurons during proestrus, pregnancy and lactation. Brain Res 654:207–215
46. **Cheung S, Will YM, Hentschel K, Moore KE, Lookingland KJ** 1997 Role of gonadal steroids in determining sexual differences in expression of Fos-related antigens in tyrosine hydroxylase-immunoreactive neurons in subdivisions of the hypothalamic arcuate nucleus. Endocrinology 138:3804–3810
47. **Ben-Jonathan N, Hnasko R** 2001 Dopamine as a prolactin (PRL) inhibitor. Endocr Rev 22:724–763
48. **Neal-Perry G, Lebesgue D, Lederman M, Shu J, Zeevalk GD, Etgen AM** 2009 The excitatory peptide kisspeptin restores the luteinizing hormone surge and modulates amino acid neurotransmission in the medial preoptic area of middle-aged rats. Endocrinology 150:3699–3708
49. **Kipp M, Karakaya S, Pawlak J, Araujo-Wright G, Arnold S, Beyer C** 2006 Estrogen and the development and protection of nigrostriatal dopaminergic neurons: concerted action of a multitude of signals, protective molecules, and growth factors. Front Neuroendocrinol 27:376–390
50. **Kauffman AS, Gottsch ML, Roa J, Byquist AC, Crown A, Clifton DK, Hoffman GE, Steiner RA, Tena-Sempere M** 2007 Sexual differentiation of Kiss1 gene expression in the brain of the rat. Endocrinology 148:1774–1783
51. **Maurer RA** 1982 Estradiol regulates the transcription of the prolactin gene. J Biol Chem 257:2133–2136
52. **West B, Dannies PS** 1980 Effects of estradiol on prolactin production and dihydroergocryptine-induced inhibition of prolactin production in primary cultures of rat pituitary cells. Endocrinology 106:1108–1113
53. **Livingstone JD, Lerant A, Freeman ME** 1998 Ovarian steroids modulate responsiveness to dopamine and expression of G-proteins in lactotropes. Neuroendocrinology 68:172–179
54. **Lee DK, Nguyen T, O'Neill GP, Cheng R, Liu Y, Howard AD, Coulombe N, Tan CP, Tang-Nguyen AT, George SR, O'Dowd BF** 1999 Discovery of a receptor related to the galanin receptors. FEBS Lett 446:103–107
55. **Kinoshita M, Tsukamura H, Adachi S, Matsui H, Uenoyama Y, Iwata K, Yamada S, Inoue K, Ohtaki T, Matsumoto H, Maeda K** 2005 Involvement of central metastin in the regulation of preovulatory luteinizing hormone surge and estrous cyclicity in female rats. Endocrinology 146:4431–4436
56. **Grattan DR, Steyn FJ, Kokay IC, Anderson GM, Bunn SJ** 2008 Pregnancy-induced adaptation in the neuroendocrine control of prolactin secretion. J Neuroendocrinol 20:497–507
57. **Smith JT, Rao A, Pereira A, Caraty A, Millar RP, Clarke IJ** 2008 Kisspeptin is present in ovine hypophysial portal blood but does not increase during the preovulatory luteinizing hormone surge: evidence that gonadotropes are not direct targets of kisspeptin *in vivo*. Endocrinology 149:1951–1959
58. **Hashizume T, Saito H, Sawada T, Yaegashi T, Ezzat AA, Sawai K, Yamashita T** 2010 Characteristics of stimulation of gonadotropin secretion by kisspeptin-10 in female goats. Anim Reprod Sci 118: 37–41
59. **Smith MS, Freeman ME, Neill JD** 1975 The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. Endocrinology 96: 219–226
60. **Ben-Jonathan N, LaPensee CR, LaPensee EW** 2008 What can we learn from rodents about prolactin in humans? Endocr Rev 29:1–41
61. **Horikoshi Y, Matsumoto H, Takatsu Y, Ohtaki T, Kitada C, Usuki S, Fujino M** 2003 Dramatic elevation of plasma metastin concentrations in human pregnancy: metastin as a novel placenta-derived hormone in humans. J Clin Endocrinol Metab 88:914–919
62. **Roa J, Vigo E, Castellano JM, Navarro VM, Fernández-Fernández R, Casanueva FF, Dieguez C, Aguilar E, Pinilla L, Tena-Sempere M** 2006 Hypothalamic expression of KiSS-1 system and gonadotropin-releasing effects of kisspeptin in different reproductive states of the female rat. Endocrinology 147:2864–2878
63. **Smith JT, Li Q, Pereira A, Clarke IJ** 2009 Kisspeptin neurons in the ovine arcuate nucleus and preoptic area are involved in the preovulatory luteinizing hormone surge. Endocrinology 150:5530–5538