RFamide-Related Peptide-3 Receptor Gene Expression in GnRH and Kisspeptin Neurons and GnRH-Dependent Mechanism of Action

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RFamide-related peptide-3 (RFRP-3) is known to inhibit the activity of GnRH neurons. It is not yet clear whether its G protein-coupled receptors, GPR147 and GPR74, are present on GnRH neurons or on afferent inputs of the GnRH neuronal network or whether RFRP-3 can inhibit gonadotropin secretion independently of GnRH. We tested the following: 1) whether GnRH is essential for the effects of RFRP-3 on LH secretion; 2) whether RFRP-3 neurons project to GnRH and rostral periventricular kisspeptin neurons in mice, and 3) whether *Gpr147* and *Gpr74* are expressed by these neurons. Intravenous treatment with the GPR147 antagonist RF9 increased plasma LH concentration in castrated male rats but was unable to do so in the presence of the GnRH antagonist cetrorelix. Dual-label immunohistochemistry revealed that approximately 26% of GnRH neurons from male and diestrous female mice were apposed by RFRP-3 fibers, and 19% of kisspeptin neurons from proestrous female mice were apposed by RFRP-3 fibers. Using immunomagnetic purification of GnRH and kisspeptin cells, single-cell nested RT-PCR, and *in situ* hybridization, we showed that 33% of GnRH neurons and 9–16% of rostral periventricular kisspeptin neurons expressed *Gpr147*, whereas *Gpr74* was not expressed in either population. These data reveal that RFRP-3 can act at two levels of the GnRH neuronal network (*i.e.* the GnRH neurons and the rostral periventricular kisspeptin neurons) to modulate reproduction but is unable to inhibit gonadotropin secretion independently of GnRH. **(*Endocrinology* 153: 3770–3779, 2012)**

Mammalian fertility is governed by the neuropeptide GnRH. In rodents, the cell bodies of GnRH neurons are scattered in the medial septum (MS) and rostral preoptic area (POA) of the forebrain. GnRH drives the pulsatile secretion of LH from the anterior pituitary gland. The gonadotropic hormones LH and FSH regulate male and female gonadal function (1, 2).

A dramatic rise in circulating estradiol concentration has been documented on the day of proestrus in females of many species (3). This high estradiol concentration stimulates a preovulatory surge in GnRH and gonadotropin release, which in turn results in ovulation. In recent years two structurally related neuropeptides that project to GnRH soma and exert opposite effects on the GnRH surge have been characterized (4–6). Kisspeptin neurons projecting from the rostral periventricular region of the third ventricle (RP3V) have been shown to provide excitatory input to GnRH neurons and are critical for surge generation in rodents (3, 7–9). In contrast, RFamide-related peptide (RFRP)-3 neurons projecting from the rodent dorsomedial hypothalamus are mostly inhibitory to GnRH activity (10, 11) and suppress the estradiol-induced

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Abbreviations: E_2, Estradiol; GFP, green fluorescent protein; GPR, G protein-coupled receptor; ISH, *in situ* hybridization; MS, medial septum; OVX, ovariectomized; POA, preoptic area; POMC, proopiomelanocortin; RFRP, RFamide-related peptide;RP3V, rostral periventricular region of the third ventricle; TBS, Tris-buffered saline; TBS-TX-BSA, TBS containing Triton X-100 and BSA.

GnRH/LH surge (12). At the time of the surge, the activity of RP3V kisspeptin neurons (assessed by c-Fos coexpression) is dramatically increased in rats and mice (9, 13), whereas c-Fos expression decreases in Syrian hamster RFRP-3 neurons at this time (14).

Virtually all GnRH neurons in rats and mice express the kisspeptin receptor, G protein-coupled receptor (GPR)-54 (also termed Kiss1r) (15–17). RFRP-1 and RFRP-3 bind with high affinity to the GPR147 and to a lesser extent to GPR74, which were first identified as neuropeptide FF receptors (in fact, RFRP-1 and RFRP-3 have a higher affinity to GPR147 than does neuropeptide FF) (18–20). The cells that express these RFRP-3 receptors remain to be characterized in the mammalian reproductive system, although there is evidence for GPR147 in rodent and starling GnRH neurons (21–23). Interestingly, we measured higher levels of *Gpr147* mRNA in the RP3V of rats than in the MS/POA (24), leading us to speculate whether RFRP-3 might act via this receptor on kisspeptin neurons in addition to GnRH neurons. RFRP-3 immunoreactive fibers are also present in the RP3V region of rats (25), and central infusion of RFRP-3 at the time of the preovulatory surge dose dependently suppresses not only GnRH neuronal activation but also activation of RP3V neurons in this species (12). *In vitro* experiments have led some investigators to suggest that RFRP-3 may also act directly on pituitary gonadotropes, but this remains to be proven *in vivo* (see Ref. 4 for review). Therefore, the aims of the current study were to test whether RFRP-3 is able to inhibit gonadotropin secretion independently of GnRH, using receptor antagonists to block the receptors for these peptides, to detect RFRP-3 fiber projections to GnRH and kisspeptin neurons in rodents, and to measure coexpression of RFRP-3 receptors on these cells.

Materials and Methods

Animals

Adult male and female Sprague Dawley rats and C57BL/6J mice (aged 8–12 wk and weighing 250–300 or 20–30 g, respectively) were obtained from the University of Otago animal breeding facility (experiments 1–4) or Harlan Laboratories (Indianapolis, IN) (experiment 5). Rodents were housed under conditions of controlled lighting (lights on from 0600 to 1800 h) and temperature (22 ± 1 C) and had free access to standard rodent chow and water. The University of Otago Animal Ethics Committee (experiments 1–4) and the Animal Care and Use Committee of the University of California, San Diego (experiment 5), approved all animal experimental protocols.

Experiment 1: GnRH dependency of RFRP-3 actions

To test whether the inhibition of LH secretion by RFRP-3 is dependent on the presence of GnRH, we used receptor antagonists to block the receptors for these peptides in castrated adult male Sprague Dawley rats. The rat model permits frequent blood collection, and the castrate is a suitable model for LH inhibition by GnRH receptor antagonism (26) but still allows some further LH stimulation by RFRP-3 receptor antagonism (our own preliminary observations). Because a direct hypophysiotropic action of RFRP-3 on the gonadotropes has been proposed (4), RF9 was administered peripherally. This enabled us to test whether RFRP-3 could suppress LH release at the pituitary level independently of GnRH.

Rats were castrated under halothane anesthesia using an open castration. One week later they were fitted with a silicone rubber atrial cannula via the right jugular vein to facilitate repeated blood sampling (12). On the morning of the following day, blood samples (0.4 ml) were collected at −30, −10, and 0 min into heparinized syringes before a bolus injection of the selective GPR147 and GPR74 antagonist RF9 [80 nmol in 0.3 ml saline, iv (0.1 mg/kg); Tocris Bioscience, Bristol, UK] or saline vehicle at 0 min. Half of the animals treated with RF9 or vehicle were pretreated with the selective GnRH receptor antagonist cetrorelix [50 nmol in 0.3 ml saline, iv (0.2 mg/kg); Anaspec, San Jose, CA] at −10 min; the remaining animals received saline vehicle (n = 5 per group). The doses of RF9 and cetrorelix were based on previous reports (26–29), and on a pilot study that showed that RF9 delivered at 0.1 and 0.2 mg/kg yielded identical LH responses (not shown). Further blood samples were collected at 10-min intervals from 0 to 30 min, at 15 min intervals from 30 to 90 min, and 30-min intervals from 90 to 150 min. Red blood cells were resuspended in sterile physiological saline and replaced into the animal following the subsequent sample. Plasma was harvested and stored at −20 C for later LH RIA as described in the Supplemental Methods, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

Experiment 2: RFRP-3 fiber close appositions with GnRH and RP3V kisspeptin neurons

We used dual immunofluorescence to quantify the percentage of GnRH neurons contacted by RFRP-3 fibers in intact males and diestrous females (n = 3 per sex), and the percentage of RP3V kisspeptin neurons contacted by RFRP-3 fibers in proestrous (midafternoon) females (n = 4). The latter animal model was chosen because immunoreactive RP3V kisspeptin soma are much more numerous when the circulating estradiol concentration is high (30). Cycle stage was determined by vaginal cytology. Mice were perfused, and the septal preoptic area and anterior hypothalamus were cut to provide three sets of consecutive sections (90 μm apart) as described in the Supplemental Methods.

All immunohistochemistry steps were separated by three washes in 0.05 M Tris-buffered saline (TBS). For RFRP-3 + GnRH immunohistochemistry, the sections were briefly blocked in TBS containing 0.5% Triton X-100 and 0.25% BSA (TBS-TX-BSA) and then incubated for 48 h at 4 C in polyclonal rabbit antirat RFRP precursor peptide antisera (GA197) (25) diluted 1:500 in TBS-TX-BSA containing 2% normal goat serum. Because the RFRP precursor peptide produces both RFRP-1 and RFRP-3 (14), labeling the precursor effectively defines RFRP-1/RFRP-3-expressing neurons. Preabsorption of the primary antiserum with either the RFRP precursor peptide sequence or mature RFRP-3 peptide resulted in a complete absence of cytoplasmic staining (25). This was followed by a 2-h incubation

with Alexa Fluor 488 goat antirabbit (1:200; Molecular Probes, Life Technologies, Grand Island, NY) in TBS-TX-BSA (this and all the subsequent steps were shielded from light to avoid photobleaching). Next, the sections were incubated for 48 h in a new polyclonal guinea pig anti-GnRH antibody (GA02; 1:3000 dilution), which was generated and characterized as described in the Supplemental Methods and Supplemental Fig. 1, followed by Alexa Fluor 568 goat anti-guinea pig (1:200; Molecular Probes) for 2 h.

Sections were mounted on glass slides and coverslipped with Vectorshield (Vector Laboratories, Burlingame, CA). Omission of GnRH and RFRP-3 primary antibodies resulted in a complete absence of staining. All GnRH neurons from the MS/POA were examined for RFRP-3 fiber close appositions on a Zeiss LSM 510 upright confocal laser scanning microscope (Carl Zeiss GmbH, Jena, Germany), with a ×63 objective, collecting images at intervals of 600 nm. The fluorochromes were detected with 488 nm (for RFRP-3) and 543 nm (for GnRH) laser excitation lines and filters. Stacks of images were examined offline using ImageJ software (National Institutes of Health, Bethesda, MD). The separately recorded green and red images were merged for analysis of close appositions, which were defined as no black pixels between the RFRP-3 fiber and the GnRH soma or a merging of green fibers onto red soma resulting in a yellow pixel. Means for each mouse were determined and grouped to provide mean ± SEM values.

For RFRP-3 + kisspeptin immunohistochemistry, the sections were stained as above except that the GnRH antisera was replaced with a polyclonal sheep anti-kisspeptin-10 antisera AC024 (31) (a kind gift from Dr. Alain Caraty, Nouzilly, France) at 1:1000 dilution, followed by Alexa Fluor 568 donkey antisheep (1:200; Molecular Probes).

Experiment 3: Gpr147 and Gpr74 mRNA in purified GnRH and RP3V kisspeptin populations

As an initial screen for the presence of *Gpr147* and *Gpr74* mRNA in GnRH and RP3V kisspeptin neurons, GnRH and kisspeptin immunolabeled cells were extracted from the brains of estradiol-treated adult female mice using an immunomagnetic separation method (MACS separation kit; Militenyl Biotec, Auburn, CA) to magnetically label cells of interest with antibiotin metallic microbeads and retain them within a magnetic field, as described previously (32).

Fresh brains were collected from two groups of intact female mice (n = 7 per group). To maximize *Kiss1* (the gene that encodes kisspeptin) gene expression within the RP3V, one group was treated with estradiol (30). These mice received two injections of β-estradiol 3-benzoate (1 μg/20 mg body weight; Sigma-Aldrich, St. Louis, MO) at 0900 h, 24 h apart, and were decapitated 24 h after the second injection. This dose of estradiol induces a preovulatory-like surge in ovariectomized female mice (33). The brain region encompassing the preoptic area and RP3V was dissected out along the following boundaries: laterally 2 mm either side of the third ventricle, rostrocaudally from the optic chiasm to 4 mm caudal to this point, and 4 mm dorsal to the base of the brain. To monitor the consistency of the amount of tissue dissected per mouse, tissue blocks were weighed. The coefficient of variation for tissue block weights was 6%.

All the following steps were performed at 4 C unless specified. The seven tissue blocks per group were immediately pooled in 1 ml of cold dispersion medium (GIBCO Hanks' balanced salt solution; Invitrogen, Life Technologies, Carlsbad, CA), minced with a scalpel blade, and centrifuged (600 × *g*). The pelleted tissues were dispersed and suspended as described in the Supplemental Methods. The cell pellet was resuspended in 2 ml of labeling buffer containing 0.5% Triton X-100 and primary antisera. The cell suspension from the non-estradiol-treated mice was incubated for 20 min at room temperature with gentle shaking in polyclonal guinea pig anti-GnRH antibody (GA02; 1:500 dilution containing 2% normal goat serum) and that from the estradiol-treated mice was incubated with polyclonal sheep anti-kisspeptin-10 antibody (AC024; 1:2000 dilution containing 2% normal donkey serum). The cells were then washed by centrifugation (300 × *g*) and resuspension of the pellet in 2 ml of labeling buffer to remove unbound primary antibody, centrifuged again, and the pellet resuspended in 160 μl of labeling buffer containing the appropriate biotinylated secondary antibody (Vector Laboratories). Goat anti-guinea pig and donkey antisheep (both 1:50 dilution) were used to label GnRH and kisspeptin cells, respectively. This was followed by the addition of 50 μl of antibiotin microbeads (Militenyl Biotec) and incubation for 15 min. The cells were then washed as before and resuspended in 1 ml of separation buffer (0.1 M PBS, pH 7.2, containing 0.5% biotin free BSA). A magnetic MidiMACS separator kit fitted with an LS column (Militenyl Biotec) was used to separate the cells, according to the cell count (>2×10^8 cells/ml). The cell suspension was added into the column and allowed to drain under gravity, and the column was washed thrice with 3 ml of separation buffer. The effluents from the suspension and washes (containing cells not bound by the GnRH and kisspeptin antibodies) were pooled. The column containing the labeled cells was removed from the magnetic separator and flushed with 5 ml of separation buffer with the aid of a plunger. The antibody-bound and antibody nonbound fractions were stored at −80 C until required for RT-PCR.

For RT-PCR, the total RNA was extracted and then reverse transcribed as described in the Supplemental Methods. PCR was then performed using 2 μl of the reverse transcriptase product in 25-μl reactions containing ReddyMix PCR master mix with 1.5 mM $MgCl_2$ (ABgene, Epsom, UK) and the appropriate oligonucleotide primer pairs at 800 nM concentration (*Gpr147* forward: gcc agc ctc acc ttc tct t; *Gpr147* reverse: atc ttg cat gtg gca ttg tc; GenBank accession no. XM_905368; annealing temperature: 59 C; 272 bp product; *Gpr74* forward: tgg aca gat tcc gct gtg tgg tc; *Gpr74* reverse: gca ctg gac gct gct tgc ct; GenBank accession no. NM_133192; annealing temperature: 58 C; 360 bp product). To confirm the efficacy of the antibody binding and separation, *Gnrh* and *Kiss1* mRNA were also detected in the reverse transcriptase products using the following primers and conditions: *Gnrh* forward: cac tgg tcc tat ggg ttg cgc; *Gnrh* reverse: agt gca tat aca tct tct tct gcc; GenBank accession no. NM_008145; annealing temperature: 59 C; 213 bp product; *Kiss1* forward: atg atc tcg ctg gct tct tgg; *Kiss1* reverse: ggt tca cca cag gtg cca ttt t; GenBank accession no. NM_178260; annealing temperature: 59 C; 91 bp product. The primers spanned at least one intron/exon boundary and could therefore be used to detect the presence of contaminating genomic DNA. As a negative control for the PCR, the cDNA template was replaced with water. For an additional negative control, a sample of cortex was processed as described above using the kisspeptin-10 primary antibody and subjected to PCR using the *Kiss1*, *Gpr147*, and *Gpr74* primers. The resulting

amplicons were resolved on a 2% agarose gel containing ethidium bromide. The GnRH and the kisspeptin immunomagnetic purification experiments were run at least twice to verify the results.

Experiment 4: percentage of GnRH neurons expressing Gpr147 mRNA

Based on the findings of experiment 3, analysis of *Gpr147* mRNA presence in GnRH neurons was undertaken using the highly sensitive technique of single-cell nested RT-PCR as reported previously (33), using the same individual GnRH cytosol samples collected for that study. Cytosol samples from arcuate proopiomelanocortin (POMC) neurons [again collected previously (33) and reanalyzed for the current study] were used as expected negative controls because we have observed few RFRP-3 fibers in the arcuate nucleus (25). GnRH neuron cytosols were collected from diestrous female mice (n = 3) expressing green fluorescent protein (GFP) under the control of the GnRH promoter (34). Eighteen samples containing *Gnrh* mRNA were used for *Gpr147* nested RT-PCR. A further 10 GnRH neuron cytosols were used as controls, including no reverse transcriptase (n = 7) and mock harvests in which cells were touched but cytoplasmic contents not collected (n = 3). Six samples containing *Pomc* mRNA were collected from diestrous female POMC-GFP mice (n = 2) (generated by mating mice homozygous for a CRE recombinase-dependent GFP reporter cassette [B6;129-Gt(ROSA)26Sortm2Sho/J] (35) with POMC-Cre (36) mice) and were used for *Gpr147* nested RT-PCR. A further three POMC neuron cytosols were used as no reverse transcriptase and mock harvest controls. An additional negative control included water in place of the cDNA template for the first round of amplification. Cytosols were harvested from coronal brain slices (200 μm thick) using patch electrodes and visualization under a fluorescence microscope and immediately reverse transcribed as previously described in detail (33). The resulting cDNA was stored at −20 C until multiplex nested PCR was performed. A 100-μl, first-round PCR was performed on 5 μl of the reverse transcriptase product from each individual cell, including multiplexed primers (500 nM) for *Gnrh*, *Pomc*, and *Gpr147*. A second-round PCR using 1 μl of the first-round product was performed to detect *Gnrh*, *Pomc*, and *Gpr147* mRNA in separate 20-μl reactions (primers 500 nM). *Gnrh* and *Pomc* primers and PCR conditions are described elsewhere (33). For *Gpr147* they were as follows: first-round primers as described above for experiment 3; second-round *Gpr147* forward: tca cca aca tgt tca tcc tca; second-round *Gpr147* reverse: atc ttg cat gtg gca ttg tc (same as first round reverse primer); GenBank accession no. XM_905368; annealing temperature: 59 C; 123 bp product. The primers spanned at least one intron/exon boundary and could therefore be used to detect the presence of contaminating genomic DNA. Resulting amplicons were resolved on a 2% agarose gel containing ethidium bromide.

Experiment 5: percentage of kisspeptin neurons expressing Gpr147 mRNA

Following on from the results of experiment 3, *in situ* hybridization (ISH) was used to determine the percentage of RP3V kisspeptin neurons expressing *Gpr147* mRNA. Adult female mice were either left gonadally intact or bilaterally ovariectomized (OVX) and implanted sc with a silicone rubber capsule (internal diameter 1.47 mm, external diameter 1.96 mm) packed with 4 mm of estradiol (E_2; diluted 1:4 with cholesterol) (n = 4 per group), as previously described (37). These implants produce elevated physiological levels of E_2 and induce high levels of *Kiss1* in the RP3V of adult mice (38–41). One week after ovariectomy, animals were anesthetized with isoflurane and rapidly decapitated. Brains were collected, frozen on dry ice, and stored at −80 C. Gonadally intact adult females were killed in diestrus, as determined by vaginal cytology. Five coronal series of 20-μm brain sections were cut on a cryostat, thaw mounted onto Superfrost-plus slides, and stored at −80 C until ISH.

Double-label ISH was performed as previously described (13, 38–40, 42) using a validated digoxigenin-labeled antisense *Kiss1* probe (1:500) (42) and a radiolabeled (^{33}P) *Gpr147* (0.05 pmol/ml) antisense probe designed to bases 246–724 of the published mouse *Gpr147* mRNA (GenBank accession no. NM_001177511). ISH slides were analyzed with an automated image processing system (Dr. Don Clifton, University of Washington, Seattle, WA) by a person unaware of the treatment group of each slide (43). Digoxigenin-labeled cells (*Kiss1* cells) were identified under fluorescence microscopy and the grain-counting software was used to quantify silver grains (representing *Gpr147* mRNA) overlying each cell. Signal to background ratios for individual cells were calculated, and a cell was considered double labeled if its ratio was greater than 3. Sense probes for *Gpr147* revealed no staining in the brains of adult female mice.

Statistical analysis

Statistical comparisons between two groups (experiments 2 and 5) were made using a Student's *t* test. The effects of RF9 and cetrorelix on plasma LH concentration (experiment 1) were determined by comparing postdrug injection values to the pooled pretreatment values using a repeated-measures, one-way ANOVA (with sampling time as the within subjects factor). Data were analyzed in their untransformed state after first checking for normality and homogeneity of variance.

Results

Experiment 1: GnRH dependency of RFRP-3 actions

As expected, treatment with RF9 alone evoked a 40% increase in mean plasma LH concentration at 20 and 30 min after the injection ($P < 0.05$ compared with pooled pretreatment values), whereas cetrorelix alone decreased plasma LH concentration to 5–36% of pretreatment levels between 20 and 160 min after the injection ($P < 0.01$). Strikingly, RF9 was completely unable to stimulate LH secretion in the presence of the GnRH antagonist, with LH concentrations mirroring those of rats treated with cetrorelix alone (plasma LH concentration suppressed to 6–35% of pretreatment levels between 20 and 160 min after the cetrorelix injection) ($P < 0.01$; Fig. 1). Plasma LH concentrations from RF9 and cetrorelix-cotreated rats were significantly lower than RF9-only-treated rats from time 10 min onward until the end of the experiment (P = 0.03–0.001).

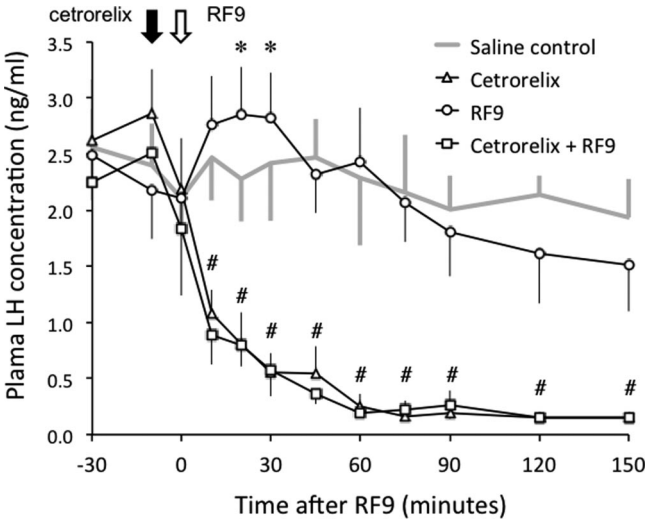

FIG. 1. Plasma LH response to treatment with the GPR147 antagonist RF9, showing that RFRP-3 is unable to inhibit gonadotropin secretion independently of GnRH. RF9 (80 nmol iv; *open arrow*) was administered either with (*squares*) or without (*circles*) pretreatment with the GnRH receptor antagonist cetrorelix (50 nmol; *filled arrow*) in castrated male rats. The stimulatory effect of RF9 was not seen when the GnRH receptor was blocked, as evidenced by the low LH levels that closely matched those of cetrorelix-only treated rats (*triangles*). Saline-treated control values are shown as a *gray line*. *, Significant stimulatory effect of RF9 alone compared with pooled pretreatment values ($P < 0.05$; repeated measures ANOVA); #, significant inhibitory effect of cetrorelix alone or of RF9 + cetrorelix compared with pooled pretreatment values ($P < 0.01$; repeated measures ANOVA). *Error bars* denote SEM.

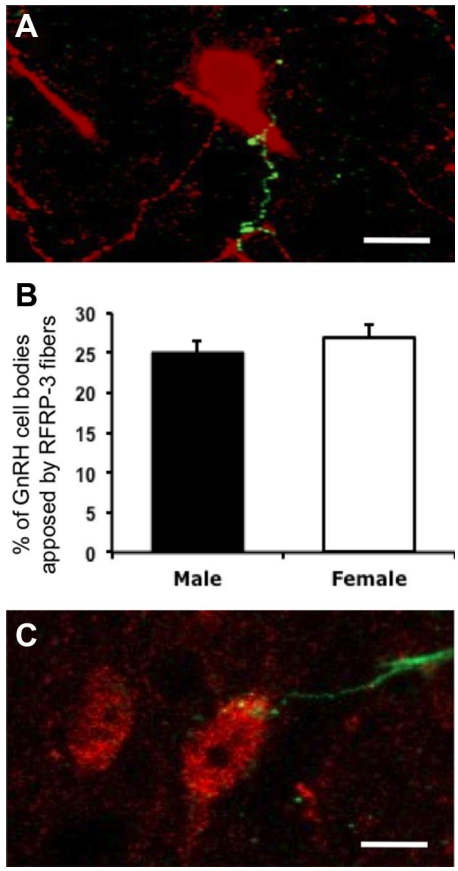

FIG. 2. Representative images of immunoreactive RFRP-3 fibers (*green fluorescence*) in close apposition with GnRH (A) and RP3V kisspeptin (C) neurons (*red fluorescence*). Figures are collapsed stacks of several confocal optical sections. There was no significant difference in the percentage of GnRH neurons from male and diestrous female mice that were apposed by RFRP-3 fibers (B). In proestrous female mice, 19% of kisspeptin neurons were apposed by RFRP-3 fibers. *Scale bars*,10 μm. *Error bars* denote SEM

Experiment 2: RFRP-3 fiber close appositions with GnRH and RP3V kisspeptin neurons

Immunoreactive RFRP-3 fibers were plentiful in the MS and rostral POA, and many of these were in apparent apposition to GnRH soma when examined at the confocal light microscopy level. In adult male and diestrous female mice, 25 and 27% of GnRH neurons analyzed showed close contact with RFRP-3 fibers, respectively (Fig. 2, A and B). There was no significant difference in the percentage of RFRP-3 fiber close appositions between males and females. In adult proestrous females, 19 ± 2.1% of RP3V kisspeptin neurons analyzed showed close contact with RFRP-3 fibers (Fig. 2C).

Experiment 3: *Gpr147* and *Gpr74* mRNA in purified GnRH and RP3V kisspeptin populations

Confirming the efficacy of the immunomagnetic cell type purification, the purified GnRH and kisspeptin cell preparations contained *Gnrh* and *Kiss1* mRNA, respectively, whereas the nonbound eluted fractions did not (Fig. 3). *Gpr147* mRNA was readily detectable by RT-PCR in the purified GnRH and kisspeptin cell preparation (Fig. 3). *Gpr147* mRNA was also present in the nonbound eluted fraction from the kisspeptin cell preparation, indicating *Gpr147* gene expression on nonkisspeptin anterior hypothalamic cells but was undetectable in the nonbound eluted fraction from the GnRH cell preparation. Negative controls included cortex subjected to kisspeptin antibody purification and water in place of cDNA template; none of these showed any products. In contrast to *Gpr147*, *Gpr74* mRNA was not detected in any of the samples. To confirm the efficacy of the *Gpr74* primers, a sample of whole hypothalamus (not subjected to the antibody treatment and separation procedure) was included as a positive control. *Gpr74* mRNA was present in this control sample. Repetition of the GnRH and kisspeptin immunomagnetic purification experiments produced identical results. The purity of the GnRH bound fraction was confirmed by the virtual absence of leptin receptor mRNA in this fraction, as previously reported for GnRH neurons (33) (Supplemental Fig. 2).

Experiment 4: percentage of GnRH neurons expressing *Gpr147* mRNA

Six of the 18 GnRH neurons (33%) confirmed as *Gnrh* positive showed the presence of *Gpr147* mRNA. Controls

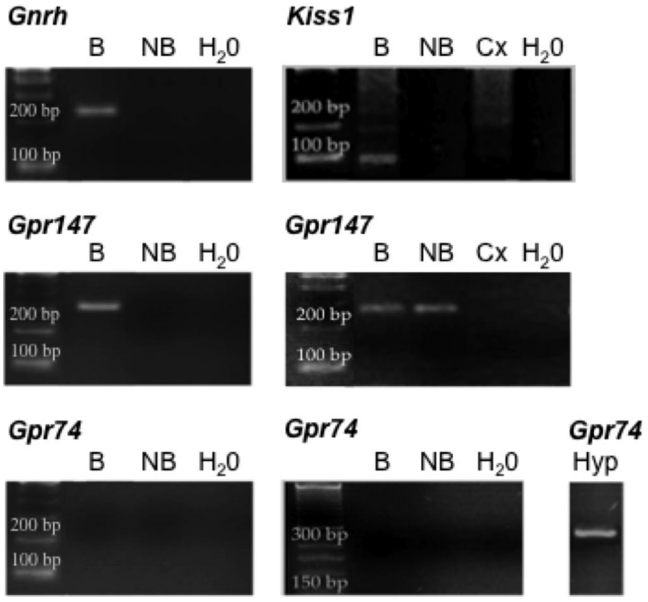

FIG. 3. Agarose gel showing RT-PCR amplicons for *Gnrh* or *Kiss1* mRNA (*upper panels*), *Gpr147* mRNA (*middle panels*), and *Gpr74* mRNA (*lower panels*). Anterior hypothalamic cell lysates were treated with a GnRH (*left panels*) or kisspeptin (*right panels*) antibody, conjugated to metallic beads, and magnetically retained on a separating column (bound cells; B), whereas the nonbound cells (NB) were eluted. Negative controls included cortex treated with the kisspeptin antibody (Cx) and water in place of cDNA template (H_2O). For *Gpr74*, a sample of whole hypothalamus (not separated as above) was run as a positive control (Hyp). The expected amplicon sizes are: *Gnrh*, 213 bp; *Kiss1*, 91 bp; *Gpr147*, 272 bp; and *Gpr74*, 360 bp. Molecular weight markers are shown in the *left lanes*, with sizes indicated in base pairs above the appropriate band.

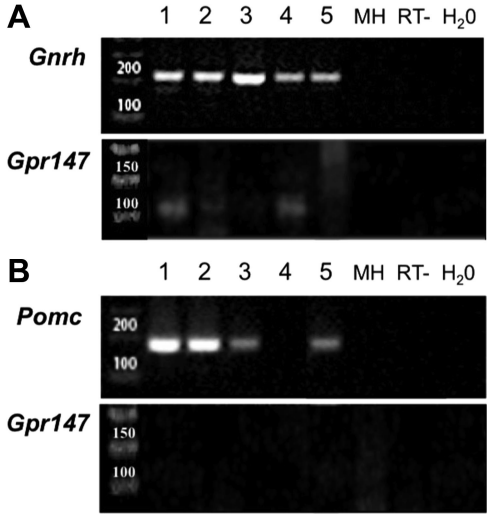

FIG. 4. Agarose gel showing representative amplicons from two rounds of nested RT-PCR using cytosols contents from individual GFP-labeled GnRH or POMC neurons. A, Cytosols from single neurons of GFP under the control of the GnRH promoter (lanes 1–5) were harvested and subjected to nested RT-PCR with primers recognizing *Gnrh* (*upper panel* of A) or *Gpr147* (*lower panel* of A) mRNA. Positive bands for *Gpr147* are shown in lanes 1, 2, and 4. B, Cytosols from single POMC-GFP neurons of the arcuate nucleus (lanes 1–5; lane 4 depicts a cell that was negative for *Pomc*) were processed as above with primers recognizing *POMC* (*upper panel* of B) or *Gpr147* (*lower panel* of B) mRNA. Negative controls (all panels), processed in parallel, included either reactions without reverse transcriptase (RT−), mock harvest controls in which the cell was touched but cytoplasmic contents were not collected (MH), or water was in place of template for the first round of amplification (H_2O). *Gpr147* transcripts were detected in 33% of GnRH neurons but not in any POMC neurons. Left lanes contain molecular weight markers, with sizes indicated in base pairs above the appropriate band. The expected amplicon sizes are: *GnRH*, 213 bp; *POMC*, 173 bp; and *Gpr147*, 123 bp.

included no reverse transcriptase, water in place of template, and mock harvests; none of these showed any product (Fig. 4A). *Gpr147* mRNA was not present in any of the six arcuate POMC neurons confirmed as *Pomc* positive; a cell type collected and processed as a likely negative control based on the sparse RFRP-3 fiber presence in the arcuate nucleus. No *Gpr147* mRNA was detected in mock harvest, minus reverse transcriptase or water controls from the POMC neurons (Fig. 4B). All the samples were analyzed in the same RT-PCR run.

Experiment 5: percentage of kisspeptin neurons expressing *Gpr147* mRNA

Using double-label ISH, we assessed the degree of coexpression between *Kiss1* and *Gpr147* in the RP3V of diestrous and OVX+E2 females. Significantly more *Kiss1* neurons were detected in OVX+E_2 females than in diestrous females, as expected based on the ability of estradiol to up-regulate *Kiss1*. In both groups, a small percentage of RP3V *Kiss1* neurons coexpressed *Gpr147* mRNA (Fig. 5A). On average, 16% of *Kiss1* neurons expressed *Gpr147* in diestrous females, whereas *Gpr147* was detected in 9% of *Kiss1* neurons in OVX+E_2 females. The percent coexpression of *Gpr147* and *Kiss1* was significantly different between diestrous and OVX+E_2 females ($P < 0.05$; Fig. 5C). In both groups, there was evidence of non-*Kiss1*-expressing cells containing *Gpr147* mRNA within the RP3V, and *Gpr147* was also strongly expressed in the dorsal lateral septum and paraventricular nucleus (Fig. 5, A and B), sites previously shown to express *Gpr147* in rodents.

Discussion

The aims of this study were to determine whether GnRH is essential for the effects of RFRP-3 on LH secretion and whether RFRP-3 neurons could act directly on GnRH and RP3V kisspeptin neurons via the receptors GPR147 and GPR74. We show here that GnRH is a critical requirement for inhibition of LH by RFRP-3. Our data also reveal that RFRP-3 fibers are apposed to subpopulations of GnRH and RP3V kisspeptin neurons and that similar proportions of these neurons express the *Gpr147* but not the

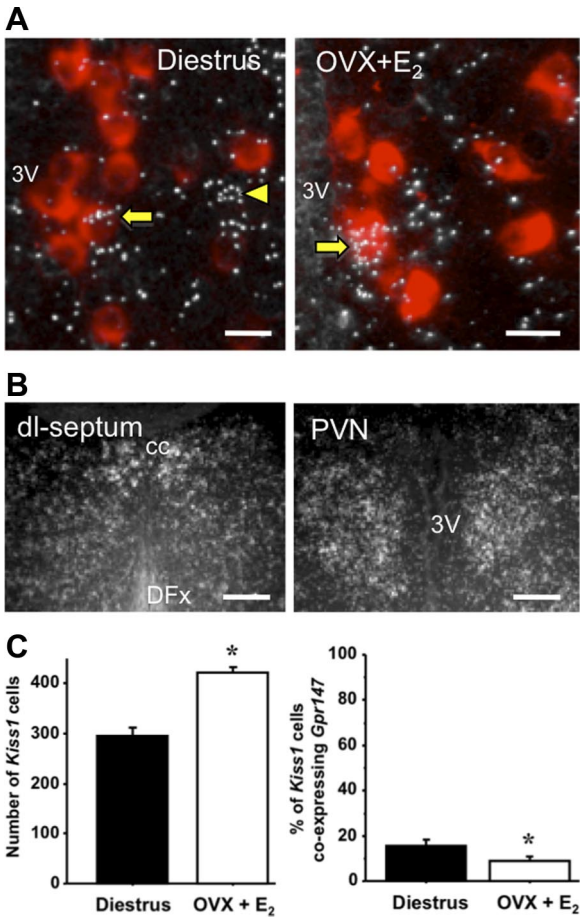

FIG. 5. Expression of *Gp147* in *Kiss1* neurons in the RP3V by *in situ* hybridization. A, Representative photomicrographs of double-label ISH of *Kiss1* (*red fluorescence*) and *Gpr147* (*silver grains*) in diestrous (*left panel*) and OVX+E_2 (*right panel*) females. Examples of *Kiss1* neurons coexpressing *Gpr147* are denoted by *yellow arrows*. Examples of *Gpr147* cells that are not *Kiss1* neurons are denoted by *yellow arrowheads*. 3V, Third ventricle. B, Low-power magnification photomicrograph of extensive *Gpr147* expression (*silver grains*) in the dorsal lateral (dl) septum (*left panel*) and paraventricular nucleus (PVN; *right panel*). cc, Corpus callosum; DFx, dorsal fornix. C, Quantification of the number of RP3V *Kiss1* neurons (*left panel*) and the percent of coexpression of *Gpr147* in *Kiss1* neurons (*right panel*) in diestrous and OVX+E_2 females. *, $P < 0.05$. *Error bars* denote SEM

Gpr74 gene. Thus, RFRP-3 is positioned to influence GnRH function both directly and indirectly, a capability that may fine-tune the animal's ability to suppress or correctly time neuroendocrine reproductive events.

The actions of RFRP-3 via GnRH neurons are well accepted, but the evidence for a hypophysiotropic effect directly on the gonadotropes is less certain because of variable reports of fiber presence in the neurosecretory external zone of the median eminence and responses to peripherally administered peptide (4). In this study we examined whether the actions of RFRP-3 were dependent on the presence of GnRH, using the GnRH receptor antagonist cetrorelix to block GnRH effects. This approach has been used to show that the stimulation of LH secretion by kisspeptin is GnRH dependent (26). However it would not be possible to demonstrate suppression of LH levels such as RFRP-3 might be expected to cause (44–46) in the presence of cetrorelix, which itself suppresses circulating LH concentrations to assay detection levels. Furthermore, suppression of tonic LH secretion by exogenous RFRP-3 is often not seen (12, 25, 46, 47), perhaps due to its short half-life (48) or already high endogenous levels of this peptide. Therefore, we instead tested whether the stimulatory effect of the GPR147/GPR74 antagonist RF9 could occur when GnRH was blocked by cetrorelix. RF9 has been shown to selectively bind to GPR147 and GPR74 but not to three other RFamide receptors (GPR10, GPR54, and GPR103) or the neuropeptide Y receptor subtype Y1 (29).

Our results show that the stimulatory effect of peripherally administered RF9 was completely absent when GnRH was blocked, revealing for the first time that RFRP-3 cannot act directly on the gonadotropes independently of GnRH in rodents. It is difficult to say whether this result proves that RFRP-3 effects are restricted to the central actions upstream of the GnRH neuron because it is possible that RFRP-3 can act on gonadotropes but only when GnRH signaling is also present. Indeed, experiments which have demonstrated effects of RFRP-3 on LH release from pituitary cells *in vitro* have been conducted in the presence of GnRH stimulation (44–46, 48, 49), implying cooperative intracellular signaling. Because this is true for both rat and sheep pituitary cells (44–46, 48, 49), it is unlikely that the GnRH dependency of RFRP-3 is species specific. A further consideration is that gonadal steroids may be required for GnRH-independent RFRP-3 actions on gonadotropes because our experiment was conducted in castrates. This seems unlikely, however, because RFRP-3 and RF9 appear to affect gonadotropin secretion from cell cultures more potently in the absence of estradiol (44–46, 48, 49) than in its presence (12). It could be argued that the ability of peripherally administered RF9 to stimulate LH secretion at all (albeit much less markedly than centrally delivered RF9) (27) provides evidence for direct pituitary effects, but this could also be explained by diffusion of RF9 into the brain. In this regard, however, it should be noted that Caraty *et al.* (47) were not able to measure RF9 in the cerebrospinal fluid of sheep after a similar iv dose to that used in our study. In the same paper, an experiment of similar design to ours was reported involving cotreatment of sheep with RF9 and a GnRH antagonist. Importantly, in that paper RF9 was given centrally, so the experiment was limited to testing whether RFRP-3 in the brain acts via GnRH neurons. Because RF9 was delivered peripherally in the current study, we are able

to address the GnRH dependency of possible gonadotropic actions of RFRP-3 as well.

A number of previous experiments have reported RFRP-3 fibers in close apposition to GnRH neurons, using various RFRP-3 antibodies on tissue from various species, with the proportion of GnRH neurons exhibiting close fiber contacts ranging from 10 to 75% (21, 50–55). We show here, using a well-characterized antibody (25), that 25–27% of mouse GnRH neurons appear to receive RFRP-3 contacts and that males and females are identical in this respect. This proportion is at the lower end of the range previously reported but agrees very closely with the proportion of GnRH neurons that expressed the *Gpr147* gene in experiment 4 (33%) and that were shown to be electrically inhibited by RFRP-3 (20–40%) (10). A very recent study also showed apparent GPR147 immunoreactivity in a surprising high proportion (86%) of GnRH neurons in hamsters (21). The difference in the percentage of cells expressing GPR147 in that study compared with ours may be a species difference but more likely represents the sensitivity of nested RT-PCR compared with immunohistochemistry for measurement of G protein-coupled receptors. From the majority of studies, it appears that considerably fewer GnRH neurons receive RFRP-3 inputs and express *Gpr147* than receive kisspeptin inputs and express *Gpr54* (15–17, 56).

Indeed, almost all GnRH neurons are contacted by RP3V kisspeptin neurons (56). *Gpr147* expression is relatively abundant in the RP3V (24), and RFRP-3 can suppress activation of some RP3V neurons (12, 57). These observations led us to speculate whether RFRP-3 might yet influence the wider GnRH neuron population indirectly via the RP3V kisspeptin neurons. The current data provide some support for this idea but only to a limited extent because the percentage of kisspeptin soma contacted by RFRP-3 fibers and expressing *Gpr147* was also very modest (19 and 9–16%, respectively). In both experiments 3 and 5, *Gpr147* mRNA was also evident in nonkisspeptin cells in the RP3V. This supports a recent report (57) that showed that administration of RFRP-3 inhibited the neuronal activity of only a small population of kisspeptin as well as nonkisspeptin cells in the RP3V. In contrast, *Gpr147* mRNA was not seen in non-GnRH cells in the nonbound fraction from experiment 3, even though the boundaries of the tissue block were similar to that used for kisspeptin immunopurification. One explanation for this is that the main concentration of *Gpr147* mRNA in the POA is within the GnRH neurons, such that the nonpurified cells in the unbound fraction (including kisspeptin cells) were collectively below the detection limit of our PCR. The generally low level of *Gpr147* expression throughout the mouse POA (23) supports this.

Because RFRP-3 also binds with moderate affinity to another closely related receptor, GPR74 (18–20), it was important to check whether GnRH or RP3V kisspeptin neurons might also have this receptor and therefore possess an additional means of responding to RFRP-3. We used an immunomagnetic purification technique to screen for the presence of *Gpr147* and *Gpr74* mRNA in GnRH and RP3V kisspeptin neurons (32). In contrast to *Gpr147* mRNA, which was present in both cell types, *Gpr74* mRNA was not detectable in either population or indeed in any POA and RP3V cells. Supporting the absence of this receptor on GnRH neurons, in a recent report, *Gpr74* mRNA was undetectable in GnRH neurons using ISH (23). The efficacy of *Gpr74* mRNA detection in our study was confirmed by the ability to detect very low levels of this transcript in a sample of whole hypothalamus (Fig. 3). Previous work has confirmed that *Gpr74* mRNA, although being moderately expressed in throughout the thalamus and lateral hypothalamus, is scarce or undetectable using ISH analysis in other regions of the rodent hypothalamus (23, 58). Our data may therefore indicate GPR147 to be the sole hypothalamic modulator of RFRP-3 actions on the reproductive axis.

We have shown that RFRP-3 cells have the potential to influence a relatively small subpopulation of GnRH neurons either directly or indirectly via RP3V kisspeptin neurons. Having both direct and indirect regulation may provide the animal with increased fidelity to control the preovulatory GnRH/LH surge independently from tonic GnRH pulses. Recent findings point to a role for RFRP-3 in preovulatory surge modulation. Central infusion of RFRP-3 at the time of the surge dose dependently suppresses both GnRH neuronal activation and activation of RP3V neurons (12). RFRP-3 neuronal activity declines at the time of the surge (14), as does the hypothalamic concentration of RFRP-3 peptide (Rizwan, M. Z., and G. M. Anderson, unpublished data). Collectively, these findings point toward a reduction in the inhibitory tone of the RFRP-3/GPR147 system as possibly being part of the mechanism for initiating increased RP3V kisspeptin and GnRH activity and hence the GnRH/LH surge.

In summary, RFRP-3 fibers project to a subpopulation of GnRH and RP3V kisspeptin neurons and RFRP-3 is unable to inhibit gonadotropin secretion independently of GnRH. In addition, *Gpr147* but not *Gpr74* mRNA is present on some GnRH and RP3V kisspeptin neurons. These data show that the RFRP-3 neuronal system regulates reproductive activity by acting at two levels of the GnRH neuronal network: the GnRH and the RP3V kisspeptin neurons.

Acknowledgments

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