Characterization of the Potent Gonadotropin-Releasing Activity of RF9, a Selective Antagonist of RF-Amide-Related Peptides and Neuropeptide FF Receptors: Physiological and Pharmacological Implications

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Identification of RF-amide-related peptides (RFRP), as putative mammalian orthologs of the avian gonadotropin-inhibitory hormone, has drawn considerable interest on its potential effects and mechanisms of action in the control of gonadotropin secretion in higher vertebrates. Yet, these analyses have so far relied mostly on indirect approaches, while direct assessment of their physiological roles has been hampered by the lack of suitable antagonists. RF9 was recently reported as a selective and potent antagonist of the receptors for RFRP (RFRPR) and the related neuropeptides, neuropeptide FF (NPFF) and neuropeptide AF (NPFF receptor). We show here that RF9 possesses very strong gonadotropin-releasing activities *in vivo*. Central administration of RF9 evoked a dose-dependent increase of LH and FSH levels in adult male and female rats. Similarly, male and female mice responded to intracerebroventricular injection of RF9 with robust LH secretory bursts. In rats, administration of RF9 further augmented the gonadotropin-releasing effects of kisspeptin, and its stimulatory effects were detected despite the prevailing suppression of gonadotropin secretion by testosterone or estradiol. In fact, blockade of estrogen receptor-α partially attenuated gonadotropin responses to RF9. Finally, systemic administration of RF9 modestly stimulated LH secretion *in vivo*, although no direct effects in terms of gonadotropin secretion were detected at the pituitary *in vitro*. Altogether, these data are the first to disclose the potent gonadotropin-releasing activity of RF9, a selective antagonist of RFRP (and NPFF) receptors. Our findings support a putative role of the RFRP/gonadotropin-inhibitory hormone system in the central control of gonadotropin secretion in mammals and have interesting implications concerning the potential therapeutic indications and pharmacological effects of RF9. **(*Endocrinology* 151: 1902–1913, 2010)**

Pituitary gonadotropins LH and FSH, as essential factors for gonadal development and function, are under the stimulatory control of GnRH, a hypothalamic decapeptide secreted into the portal system by a scarce cell population (namely, GnRH neurons), located in the basal forebrain (1–3). This neuronal system is considered as the final integrator and major output pathway for the neural regulation of gonadotropin synthesis and release and, as such, the ultimate target of a wide diversity of modulatory signals, of peripheral and central origin, with either exci-

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Abbreviations: AUC, Area under the curve; D-1, diestrus-1; E2, estradiol-17β; ER, estrogen receptor; GnIH, gonadotropin-inhibitory hormone; icv, intracerebroventricular; Kp-10, kisspeptin-10; NPFF, neuropeptide FF; NPFFR, NPFF receptor; RFa, RF-amide; RFRP, RFa-related peptide; RFRPR, RFRP receptor; T, testosterone.

tatory or inhibitory effects (1, 4, 5). Characterization of the afferent pathways and major regulators of GnRH neurons has drawn considerable attention in recent years, and significant progress has been made in elucidation of the origin (*e.g.* neuronal *vs.* glial), nature (*e.g.* kisspeptins) and mechanisms of action (*e.g.* direct contact *vs.* indirect afferents) of a plethora of modulatory signals (1, 3–7).

In the search for novel regulators of the gonadotropic axis, a 12-amino-acid neuropeptide, with a distinctive RF-amide (RFa) motif at its C terminus, was isolated in 2000 from the avian brain and termed gonadotropin-inhibitory hormone (GnIH), by virtue of its ability to suppress gonadotropin release in quail pituitaries (8). Independently, RFa-related peptides (RFRP)-1 and -3 were cloned also in 2000 as the products of the human gene *RFRP* (9) and shown to have sequence homology with neuropeptides FF (NPFF) and AF (NPAF), additional representatives of the limited family of RFa peptides in mammals. The biological actions of these peptides are mediated via distinct but related surface receptors; RFRP-1 and RFRP-3 are preferential ligands of RFRP receptor (RFRPR; also termed NPFF1R or GPR147), whereas NPFF and NPAF bind and activate NPFF receptor (NPFFR; also termed NPFF2R) (10–13). For the sake of homogeneity, in the present work, we will adopt the nomenclature RFRPR and NPFFR.

Although the biological actions of NPFF have been thoroughly characterized, and this peptide had been involved in a variety of functions, including modulation of opioid transmission and nociception (14–17), the roles of RFRPs remained poorly documented until 2006, when Kriegsfeld and colleagues (18) proposed them as the mammalian counterparts of avian GnIH, thus putatively playing a role in the inhibitory control of gonadotropin secretion in higher vertebrates. This boosted a considerable interest in the field, and subsequently, the patterns of brain expression as well as the effects and mechanisms of actions of RFRPs have been deeply scrutinized in different species (13, 19–30). As a whole, the available data suggest that RFRPs are involved in the negative regulation of the gonadotropic axis in rodents and sheep (13). Yet, some controversy persists on the actual physiological relevance and major sites of action of this neuropeptide system in the control of gonadotropin secretion in mammals (13). This might be partially due to the preferential use of indirect experimental approaches (*e.g.* testing of pharmacological doses of exogenous agonists). In contrast, direct assessment of the roles of endogenous RFRPs remains largely incomplete; for instance, the consequences of acute blockade of RFRP actions have not been described to date.

Recently, RF9 was reported as a potent and selective antagonist of NPFFRs, with similar binding affinity and antagonistic activity at RFRPR and NPFFR (12). This compound was shown to effectively block the effects of NPFF on heart rate and blood pressure and to prevent opioid-induced hyperalgesia and tolerance in rats, phenomena that are presumably mediated via NPFFR (12). However, despite some recent progress in the pharmacological characterization of this compound (31–33), the potential effects of RF9 on RFRP-related functions, including, prominently, the regulation of gonadotropin secretion, remain completely unexplored. Given the putative roles of RFRPs as mammalian counterparts of GnIH (13, 18), such testing may provide relevant physiological information. Moreover, considering the proposed use of RF9 as therapeutic agent (*e.g.* in the management of chronic pain) (12, 31, 32), assessment of gonadotropin responses to the antagonist appears mandatory from a pharmacological perspective as a means to define the whole spectrum of biological effects of this compound.

To document the potential physiological importance of orthologs of GnIH in the control of gonadotropin secretion in mammals, we explored the effects of administration of RF9 on the circulating levels of LH and FSH in rodents. Our studies included thorough characterization of the responses of both gonadotropins to administration of the antagonist in different species (rat and mouse), over a wide range of doses, through different routes, and in different functional states of the gonadotropic axis. In addition, the influence of other key regulators of the gonadotropic axis, such as kisspeptin and sex steroids, on gonadotropin responses to RF9 was also evaluated.

Materials and Methods

Animals and drugs

Wistar rats and C57BL6/J mice bred in the vivarium of the University of Córdoba were used. The animals were maintained under constant conditions of light (14 h light, from 0700 h) and temperature (22 C) and housed in groups of four rats per cage until the beginning of the pharmacological tests, with free access to pelleted food and tap water. Experimental procedures were approved by the Córdoba University Ethical Committee for animal experimentation and conducted in accordance with the European Union normative for care and use of experimental animals. RF9, antagonist of RFRPR and NPFFR, and the antagonist of estrogen receptor (ER)α, ERA-90, were generously supplied by Schering-Plough (Oss, The Netherlands). The chemical structure of RF9 is presented in Supplemental Fig. 1 (published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). Various batches of RF9, with purities varying from 85–95%, were used for the *in vivo* experiments; batches were synthesized in-house by a third party. Rat/mouse KiSS-1 (110–119)-NH_2, termed hereafter kisspeptin-10 (Kp-10), was obtained from Phoenix Pharmaceuticals (Belmont, CA). Estradiol-17β (E2) and testosterone (T) were purchased from Sigma Chemical Co. (St. Louis, MO).

Experimental designs

In the first series of experiments, the potential effects of RF9, as selective RFRPR and NPFFR antagonist, on gonadotropin secretion were evaluated in male and female rodents. As a general procedure, and to target central neuroendocrine pathways, a protocol of intracerebroventricular (icv) injection of RF9 was implemented, as described in detail elsewhere (34, 35). To allow delivery of RF9 into the lateral cerebral ventricle, the cannulae were lowered to a depth of 4 mm beneath the surface of the skull, with an insert point at 1 mm posterior and 1.2 mm lateral to bregma. In experiment 1, the effects of RF9 were assessed in adult female rats, at two different stages of the estrous cycle: estrus and diestrus-1 (D-1). To this end, adult virgin female rats were monitored for estrous cyclicity by daily vaginal cytology. Only rats with at least two consecutive regular 4-d estrous cycles were used for the subsequent studies. Pharmacological tests were conducted in groups of cycling female rats (n = 10 per group and phase), between 0900 and 1000 h (*i.e.* in the morning of corresponding stage of the cycle). A dose of 20 nmol RF9 (in 10 μl per rat) was selected on the basis of previous references (12). Blood samples (250 μl) were obtained by jugular venipuncture before (0 min) and at 15, 60, and 120 min after icv administration. Animals injected with vehicle (NaCl 0.9%) served as controls.

On the basis of initial results, in experiment 2, a detailed dose-response analysis of the effects of centrally administered RF9 was conducted in adult virgin female rats at estrus. To this end, groups of female rats (n = 10 per group) were implanted with icv cannulae as described above, and RF9 was centrally injected over a range of doses (10 and 100 pmol and 1, 5, and 20 nmol). Blood samples (250 μl) were obtained by jugular venipuncture before (0 min) and at 15, 30, 60, and 120 min after icv injections of RF9.

To evaluate the effects of RF9 in adult male rats, in experiment 3, groups of males (n = 10) were implanted with icv cannulae, as described above, and injected with RF9 over a range of doses (10 and 100 pmol and 1, 5, and 20 nmol in 10 μl). Blood samples (250 μl) were obtained by jugular venipuncture before (0 min) and at 15, 30, 60, and 120 min after icv injection.

Finally, in experiment 4, the effects of icv injection of RF9 on gonadotropin secretion were tested in adult male and female mice; the latter were taken at random stages of the estrous cycle. Groups of mice (n = 7–8 per group) were implanted with icv cannulae, with a depth of 2.5 mm beneath the surface of the skull and an insert point at 1 mm posterior and 1 mm lateral to bregma. The animals were injected with an effective dose (20 nmol) of RF9, and blood samples were taken at 15 min after administration of the compound; mice injected with vehicle served as controls. Tests were conducted between 0900 and 1000 h.

In the second series of experiments, we explored the potential interaction of RF9 with other major regulators of the gonadotropic axis, namely kisspeptins and sex steroids. In experiment 5, the combined gonadotropin-releasing effects of Kp-10 and RF9 were explored. To this end, groups of adult male rats (n = 10 per group) were implanted with icv cannulae, as described above, and injected with Kp-10 (100 pmol), RF9 (20 nmol), or Kp-10 plus RF9. The dose of Kp-10 was selected to achieve an effective but submaximal stimulation of the gonadotropic axis, in keeping with previous references (34, 35). Blood samples (250 μl) were obtained by jugular venipuncture before (0 min) and at 15, 30, 60, and 120 min after icv injection of the compounds.

In addition, in experiment 6, the potential influence of changes in gonadal steroid milieu on the gonadotropic responses to RF9 was explored in the male rat. Adult male rats (n = 10) were implanted with SILASTIC brand (Dow Corning, Midland, MI) silicon tubing elastomers (20 mm length; inner diameter, 0.079 cm; exterior diameter, 0.125 cm) containing T at a dose of 100 mg/ml dissolved in olive oil. An additional group (n = 10) of adult males was implanted with empty capsules to serve as controls. Seven days after beginning of T supplementation (or empty capsule insertion), the animals were implanted with icv cannulae and subjected to testing of gonadotropic responses to central administration of 20 nmol RF9, as described in previous experiments. Blood samples (250 μl) were obtained by jugular venipuncture before (0 min) and at 15, 60, and 120 min after icv injection of RF9.

In the same line, in experiment 7, the influence of sex steroids on the gonadotropic responses to RF9 was explored in the female rat. Groups of regularly cycling, adult virgin female rats (n = 10–12) were implanted with SILASTIC brand elastomers (20 mm length; inner diameter, 0.079 cm; exterior diameter, 0.125 cm) containing E2 at a concentration of 10 mg/ml (dissolved in olive oil). Functional testing of *in vivo* gonadotropic responses to 20 nmol RF9 was conducted at d 7 after the beginning of hormone supplementation, following a protocol similar to that of previous experiments.

Finally, in experiment 8, the impact of selective blockade of estrogen signaling, via ERα, on the gonadotropic responses to RF9 was evaluated in female rats. Given our recent findings on the consequences of acute ERα blockade on LH and FSH responses to Kp-10 at the preovulatory phase (36, 37), the experiment was conducted at this stage, using the same experimental protocol. This allowed us to comparatively reassay the effects of Kp-10 in these conditions and to monitor the gonadotropin responses after the combined administration of RF9 and Kp-10. Adult virgin female rats (n = 12 per group), checked for regular estrous cyclicity, were injected twice (at 2100 h of D-2 and 0900 h of proestrus), via sc route, with an effective dose of the selective ERα antagonist, ERA-90 (1.5 mg/kg), in keeping with previous studies (36, 37). The animals were subsequently injected at 1200 h of proestrus with a single icv injection of RF9 (20 nmol) or RF9 plus Kp-10 (1 nmol). The effects of RF9 on LH and FSH secretion in animals not injected with the ERα antagonist were also monitored for comparison. Blood samples (250 μl) were obtained by jugular venipuncture before (0 min) and at 15, 60, 120, 240, 360, and 480 min after central injection of the compounds, in line with previous studies (36, 37). Additional blood samples were taken from each animal between 0900 and 1000 h of the following estrus. For comparative purposes, LH and FSH levels were reassayed in samples from our previous study (36, 37), where the effects of Kp-10 (1 nmol; icv injection at 1200 h proestrus) were tested in animals pretreated or not with the ERα antagonist.

In the final series of experiments, we explored the effects of systemic administration of RF9 on gonadotropin secretion as well as potential direct pituitary actions of the compound. Thus, in experiment 9, RF9 (0.1 mg/kg rat) was administered via the ip route to adult male rats (n = 10); the dose of RF9 was selected on the basis of previous studies (12). Blood samples were obtained by jugular venipuncture before (0 min) and at 15, 30, 60, and 120 min after ip injection of the compound. Animals injected with vehicle (0.9% NaCl) served as controls. Finally, in experiment 10, static incubations of pituitary tissue from adult male

TABLE 1. Summary of experimental work implemented for the analysis of the gonadotropic effects of RF9

Experimental designs	Fig.
Exp. 1: Effects of RF9 on LH and FSH secretion in cycling female rats	1A and 2A
Exp. 2: Dose-response analysis of the effects of RF9 in cycling female rats	1B and 2B
Exp. 3: Dose-response analysis of the effects of RF9 in adult male rats	3
Exp. 4: Effects of RF9 on LH and FSH secretion in male and female mice	4
Exp. 5: Combined effects of RF9 and Kp-10 on gonadotropin secretion	5
Exp. 6: Influence of sex steroids on RF9 responses in male rats	6A
Exp. 7: Influence of sex steroids on RF9 responses in female rats	6B
Exp. 8: Estrogen signaling and RF9 responses in female rats	7[a]
Exp. 9: Effects of systemic administration of RF9 on gonadotropin secretion	8A
Exp. 10: Direct pituitary effects of RF9 on gonadotropin secretion *in vitro*	8B

For each experiment (Exp.), a brief descriptor is provided in keeping with its contents, as explained in detail in *Materials and Methods*. In addition, indication of the corresponding figures, where data from each experiment are presented, is also included.

[a] Data from experiment 8 are also shown in Supplemental Figs. 2 and 3.

rats were used to assess potential direct actions of RF9 at this site. Procedures for incubation of hemipituitary explants were as described in detail elsewhere (34, 35). Briefly, upon decapitation of the animals, anterior pituitaries were excised, halved (n = 12 hemipituitaries per group), and placed in scintillation vials in a Dubnoff shaker at 37 C with constant shaking (60 cycles/min) under an atmosphere of 95% O_2/5% CO_2. After 1 h preincubation, the media were replaced by either fresh medium alone (DMEM) or medium containing increasing doses of RF9 (10^{-10}, 10^{-8}, and 10^{-6} M). Samples from the incubation media were collected at 120 min for hormone determinations.

A summary presentation of all the experimental designs included in this study, with indication of corresponding figures where the experimental data from each one are presented, is provided in Table 1.

Gonadotropin measurements by specific RIAs

Serum LH and FSH levels were determined in a volume of 25–50 μl using a double-antibody method and RIA kits supplied by the National Institutes of Health (Dr. A. F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Peptide Program, Torrance, CA). Rat LH-I-10 and FSH-I-9 were labeled with ^{125}I using Iodogen tubes, following the instructions of the manufacturer (Pierce, Rockford, IL). Hormone concentrations are expressed using reference preparations LH-RP-3 and FSH-RP-2 as standards. Intra- and interassay coefficients of variation were, respectively, less than 8 and 10% for LH and less than 6 and 9% for FSH. The sensitivity of the assay was 5 pg/tube for LH and 20 pg/tube for FSH. Accuracy of hormone determinations was confirmed by assessment of rat serum samples of known concentrations (external controls).

Presentation of data and statistics

Hormonal determinations were conducted in duplicate, with a minimal total number of 10 samples per group (except for mouse experiments; n = 7–8 per group). When appropriate, integrated gonadotropin secretory responses were also calculated as the area under the curve (AUC), following the trapezoidal rule, in agreement with previous references. Results were analyzed for statistically significant differences using single or repeated ANOVA followed by Student-Newman-Keuls multiple range test (SigmaStat 2.0; Jandel Corp., San Rafael, CA). $P \leq 0.05$ was considered significant.

Results

Central injection of RF9 potently stimulates gonadotropin secretion in male and female rodents

The effects of RF9 on gonadotropin secretion were initially explored in cycling female rats (experiment 1). Based on previous reports on the hypothalamic expression and actions of RFRPs (13), a central (icv) route of administration and a dose of 20 nmol were selected. Intracerebral injection of RF9 evoked robust LH secretory responses in cycling rats at the two stages of the cycle tested, estrus and D-1, with peak values at 30 min and persistently elevated LH concentrations during the 120-min period after RF9 injection. When calculated as integrated AUC, relative LH responses to central administration of RF9 represented approximately 5.0- and 2.5-fold increases over corresponding vehicle-injected controls at estrus and D-1, respectively (Fig. 1A). Similarly, RF9 elicited significant elevations of circulating FSH levels in cycling females at estrus but not at D-1, responses whose magnitude represented only a relative increase of 1.4-fold over vehicle-injected controls (Fig. 2A).

These observations prompted us to conduct detailed dose-response analyses in female rats; assays were carried out in cycling females at estrus and involved the testing of a wide range of doses of RF9 (10 and 100 pmol and 1, 5, and 20 nmol icv) (experiment 2). Assessment of the LH-releasing effects of RF9 demonstrated consistent stimulation for doses of RF9 from 5 nmol onward, as revealed by time-course analyses, linear regression of hormonal values at 30 min after injection of RF9, and integrated (AUC) responses during the 120-min period after icv administration of the antagonist (Fig. 1B). A similar trend was detected for FSH responses to RF9 in female rats at estrus, although the magnitude of FSH responses to 5 nmol RF9 did not reach statistical significance (Fig. 2B).

Similar dose-response curves were generated in adult male rats after icv injection of different doses of RF9 and serial blood sampling over 120 min (experiment 3). As shown in Fig. 3A, consistent stimulation of LH secretion

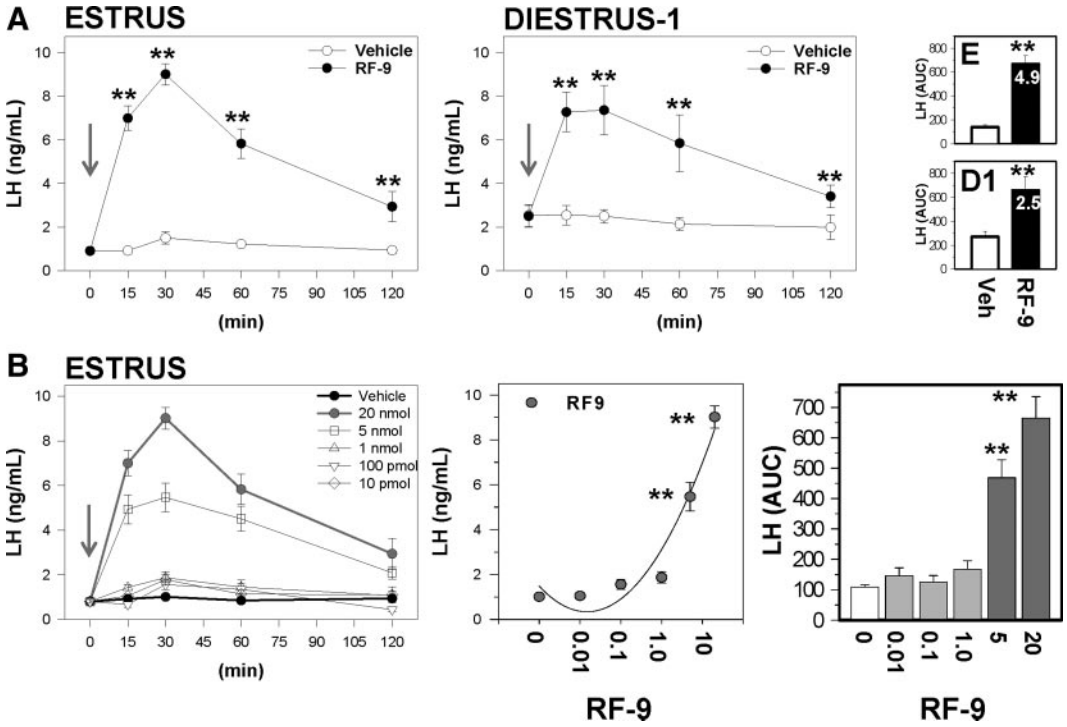

FIG. 1. Effects of icv injection of RF9 on LH secretion in cycling female rats. A, Effects of a single icv bolus of RF9 (20 nmol; denoted by an *arrow*) on LH levels in cycling females at estrus and D-1 over a period of 120 min. In addition to time-course data, integrated hormonal responses to RF9 or vehicle during the 120 min are depicted as AUC; numeric values in the histograms represent fold increases over basal levels. B, Dose-response analyses for the LH-releasing effects of RF9 in cycling female rats at estrus. In addition to time-course data, regression analyses of peak hormonal levels (at 30 min after RF9 injection) and integrated (AUC) hormonal responses during the 120 min are shown. **, $P \leq 0.01$ *vs.* control (vehicle) group (ANOVA followed by Student-Newman-Keuls multiple-range test).

was detected in male rats from doses of 1 nmol/icv RF9 onward, as estimated by time-course analyses, linear regression (levels at 30 min after RF9 injection), and integrated responses during the 120-min period after administration of the antagonist; the magnitude of LH responses to RF9 was clearly dose dependent. In contrast, only the dose of 20 nmol RF9 icv was capable of eliciting unambiguous FSH responses in male rats (Fig. 3B).

Finally, to explore for potential species differences, gonadotropin responses to RF9 (at an effective dose of 20 nmol icv) were also monitored in adult male and female mice (experiment 4). As shown in Fig. 4, RF9 elicited robust LH secretory peaks at 15 min after its icv injection, which represented a more than 20-fold increase over basal concentrations in vehicle-injected controls and whose net amplitude was similar between males and females. At this time point, however, central injection of RF9 failed to evoke significant FSH responses in female mice, whereas it elicited a modest 35% increase in serum FSH levels in adult males.

Gonadotropin responses to RF9 in rats: influence of kisspeptin and sex steroids

The gonadotropin-releasing effects of RF9 were compared with those of an effective, albeit submaximal, dose of Kp-10 (0.1 nmol) in male rats (experiment 5). In addition, the effects of coadministration of RF9 and Kp-10 were explored in the same experiment. Central injection of 0.1 nmol Kp-10 elicited the expected rise in serum LH levels, which peaked at 15 min (4-fold increase) and declined thereafter; LH concentrations at 120 min were similar to preinjection values. Likewise, icv administration of 20 nmol RF9 elicited very robust LH secretory responses with peak levels at 30 min (6-fold increase), in keeping with our original findings. Yet LH levels after RF9 were invariably higher than those after Kp-10 administration at all time points studied. Accordingly, integrated LH responses during the 120-min period after icv administration of RF9 doubled those induced by Kp-10. Notably, coinjection of RF9 and Kp-10 resulted in elevated LH levels that were similar to those observed after icv administration of RF9 alone, except for the concentrations reached at 120 min that were significantly higher after the combined injection of RF9 and Kp-10 (Fig. 5). FSH levels were also increased after Kp-10 or RF9 administration; FSH concentrations were higher in rats injected with RF9 at 60 and 120 min. Coadministration of RF9 and Kp-10 elicited FSH secretory responses higher than those of Kp-10 alone but not statistically different from those of RF9 (Fig. 5).

The ability of RF9 to stimulate LH and FSH secretion was also tested in male and female rats exposed to high

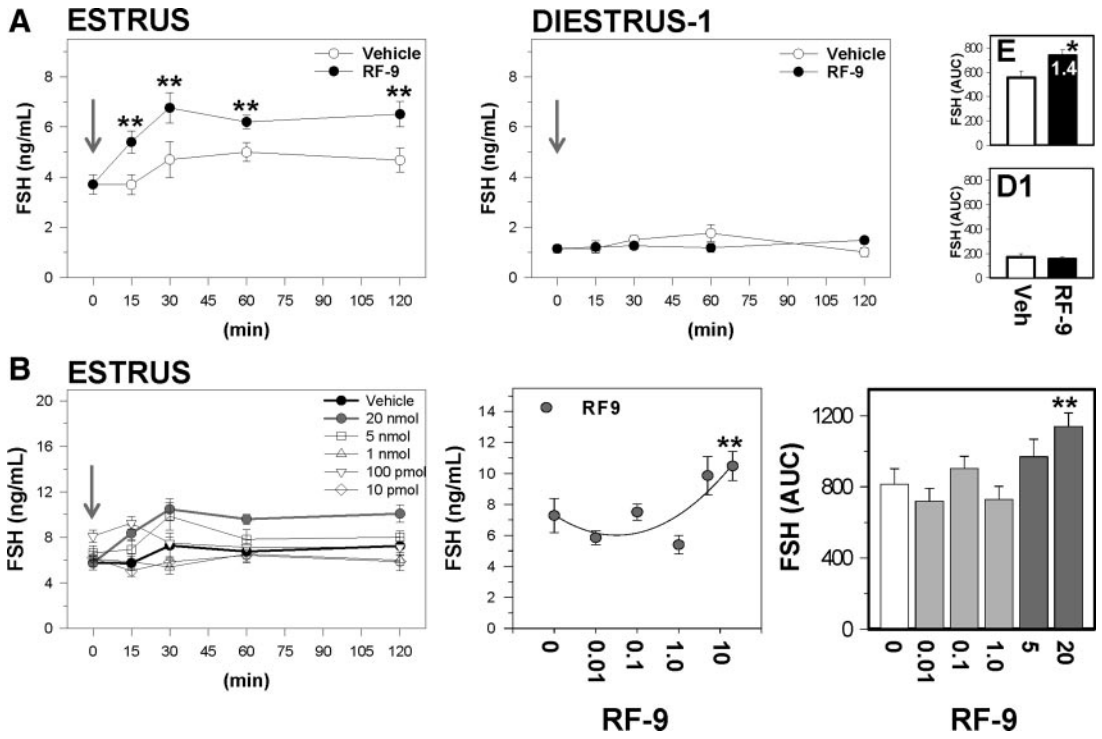

FIG. 2. Effects of icv injection of RF9 on FSH secretion in cycling female rats. A, Effects of a single icv bolus of RF9 (20 nmol; denoted by an *arrow*) on FSH levels in cycling females at estrus and D-1 over a period of 120 min. In addition to time-course data, integrated hormonal responses to RF9 or vehicle during the 120 min are depicted as AUC; numeric values in the histograms represent fold increases over basal levels. B, Dose-response analyses for the FSH-releasing effects of RF9 in cycling female rats at estrus. In addition to time-course data, regression analyses of peak hormonal levels (at 30 min after RF9 injection) and integrated (AUC) hormonal responses during the 120 min are shown. *, $P \leq 0.05$; **, $P \leq 0.01$ *vs.* control (vehicle) group (Student *t*-test and ANOVA followed by Student-Newman-Keuls multiple-range test, respectively).

levels of sex steroids (T in males and E2 in females; experiments 6 and 7, respectively) at concentrations sufficient to effectively suppress circulating gonadotropins. This setting was selected because RFRPs have been previously involved in mediating negative feedback control of the gonadotropic axis (18). In males, high T levels virtually nullified serum LH levels and significantly decreased FSH concentrations. Yet for both hormones, icv injection of RF9 was able to elicit robust and sustained elevations of their circulating levels, whose absolute magnitude was nevertheless lower than in control animals at all time points studied (except for LH concentrations at 120 min). Of note, however, due to the marked suppression of the prevailing gonadotropin concentrations, relative increases over preinjection values after RF9 injection were even augmented in T-treated males (Fig. 6A). In females, a similar phenomenon was observed after supplementation with high doses of E2; basal LH and FSH levels were decreased to nearly negligible levels, and absolute LH responses to RF9 were partially suppressed. Yet relative LH and FSH responses to RF9 were augmented; cycling females at D-1 were taken as reference controls (Fig. 6B). Contrary to males, however, such an increase was more pronounced for FSH in the case of females.

On the basis of the above observations, the influence of ERα signaling in gonadotropin responses to RF9 was explored in cycling female rats (experiment 8). This study was conducted at the preovulatory phase (see *Materials and Methods*), and the effects of Kp-10 in the same experimental paradigm are provided for comparison. In keeping with our initial findings in cycling female rats (at estrus and D-1), icv injection of a single bolus of RF9 to control females at proestrus induced a robust LH secretory response between 1200 and 1400 h, with peak levels at 1300 h. This was followed by an augmentation and shift to the left of the endogenous preovulatory surge of LH that reached maximal levels at 1800 h and declined thereafter. Blockade of ERα signaling resulted in a significant attenuation of acute LH responses to RF9 at proestrus (Fig. 7A). These profiles of LH responses were roughly similar to those induced by Kp-10 in control and anti-ERα-treated females (Fig. 7B). Coadministration of RF9 and Kp-10 to female rats treated with the antagonist of ERα elicited LH responses of higher magnitude than those evoked by any of the compounds alone, which overcame the decrease in responsiveness imposed by antagonism of ERα (Supplemental Fig. 2A). None of the treatments (RF9, Kp-10, or RF9 plus Kp-10) was able to rescue the endogenous preovulatory surge of LH in proestrous females injected with anti-ERα.

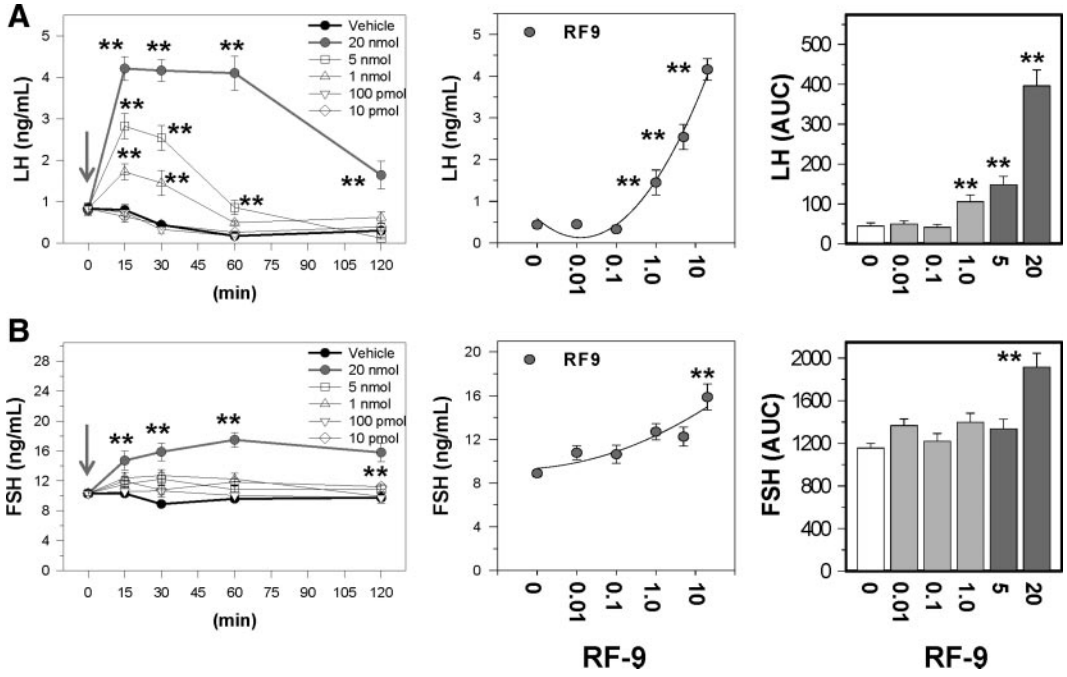

FIG. 3. Effects of icv injection of RF9 on gonadotropin secretion in adult male rats. A, Dose-response analyses for the LH-releasing effects of RF9 in adult male rats. In addition to time-course data, regression analyses of peak hormonal levels (at 30 min after RF9 injection) and integrated (AUC) hormonal responses during the 120 min are shown. B, FSH responses to RF9 in adult males. **, $P \leq 0.01$ *vs.* corresponding control group (ANOVA followed by Student-Newman-Keuls multiple-range test).

To ease comparison of LH responses to RF9 between groups, the integrated LH secretion between 1200 and 1400 h proestrus (*i.e.* after icv injection of RF9 and/or Kp-10) was calculated as AUC. This was considered as the primary response to the pharmacological agents and termed first peak in keeping with recent studies (36). In addition, the magnitude of the endogenous LH surge was estimated as AUC between 1400 and 2000 h (namely, second peak). As shown in Fig. 7C, the magnitude of the first peak in response to RF9 was attenuated in anti-ERα-treated females; coadministration of RF9 and Kp-10 reversed such attenuation (Supplemental Fig. 2B). Likewise, blockade of ERα signaling dramatically suppressed the magnitude of the endogenous LH peak after RF9 administration, a condition that was only partially reversed by coadministration of Kp-10 (Supplemental Fig. 2B). Again, these profiles of integrated LH responses were roughly similar to those elicited by Kp-10 under similar experimental conditions (Fig. 7D).

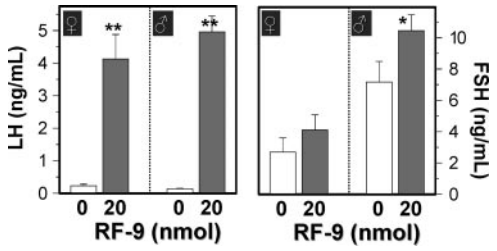

FIG. 4. Effects of icv injection of RF9 on gonadotropin secretion in adult male and female mice. The effects of a single icv bolus of RF9 (20 nmol) on LH and FSH levels in adult male and female mice are depicted. Hormonal values were assayed at 15 min after icv injection of the compound. *, $P \leq 0.05$; **, $P \leq 0.01$ *vs.* corresponding control group (Student's *t* test).

FSH responses to RF9 (alone or in combination with Kp-10) were also evaluated in the above setting. As was the case in cycling females at estrus, icv injection of RF9 to control females at 1200 h proestrus elicited an unambiguous rise in serum FSH levels, which gradually increased during the afternoon/evening of proestrus. This pattern of response to RF9 was severely blunted in females treated with the antagonist of ERα (Supplemental Fig. 3A). For quantitative comparison, integrated secretory FSH responses were calculated as AUC; yet, due to the dynamics of FSH secretion, a single peak (between 1200 and 2000 h) was considered. As shown in Supplemental Fig. 3B, FSH secretory mass in response to RF9 was significantly reduced after antagonism of ERα signaling, a phenomenon that could not be rescued by coadministration of Kp-10.

Effects of systemic administration and direct pituitary actions of RF9 on gonadotropin secretion

Finally, the effects of systemic administration of RF9 on gonadotropin secretion were explored in male rats after ip administration of 0.1 mg/kg of the antagonist (experiment 9), in keeping with previous studies of peripheral injection in models of opioid tolerance (18). Injection of a single bolus of RF9 elicited a significant increase in circulating LH levels, which peaked at 30 min and declined thereafter;

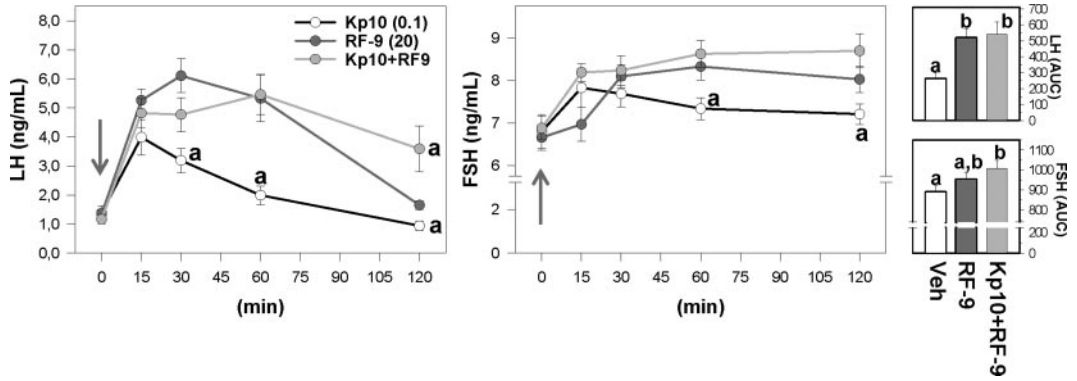

FIG. 5. Effects of RF9, Kp-10, or their coadministration on gonadotropin levels in male rats. Time-course analysis of the effects of a single icv injection of RF9 (20 nmol), Kp-10 (0.1 nmol), or their coadministration on circulating LH and FSH levels in adult male rats. Integrated hormonal responses to the different treatments during the 120 min after icv injections, calculated as AUC, are also presented. For time-course analyses: a, $P \leq 0.01$ *vs.* corresponding RF9-injected group. For AUC data, groups with *different superscript letters* are statistically different (ANOVA followed by Student-Newman-Keuls multiple-range test).

no significant elevation in LH levels was detected from 60 min after RF9 administration onward (Fig. 8A). In contrast, systemic injection of RF9, at the dose tested, failed to modify circulating FSH levels at any time point studied (data not shown). In addition, potential direct pituitary effects of RF9 were evaluated using static incubations of pituitary tissue from male rats (experiment 10). None of the doses of RF9 tested (10^{-10}, 10^{-8}, and 10^{-6} M) significantly modified LH secretion by pituitary explants *in vitro* (Fig. 8B). Similarly, pituitary FSH secretion remained unaltered after exposure to increasing concentrations of RF9 (data not shown).

Discussion

The neurohormonal systems responsible for the control of the gonadotropic axis in mammals have been deeply ex-

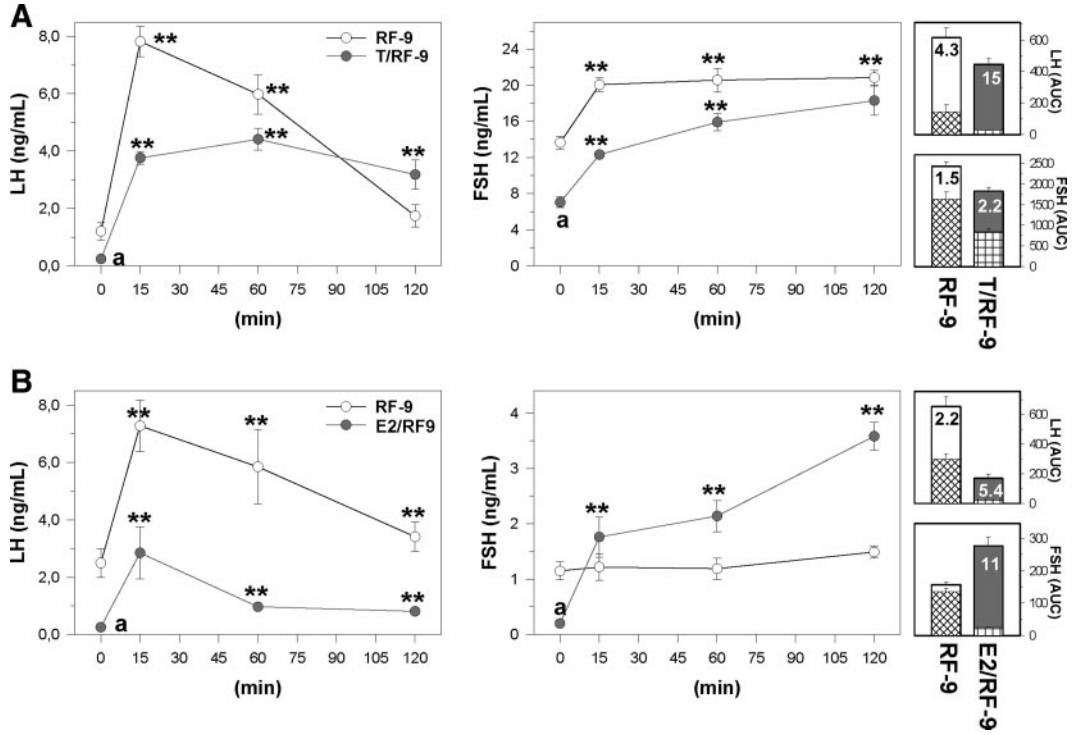

FIG. 6. Effects of icv injection of RF9 on gonadotropin secretion in sex steroid-treated rats. A, Effects of a single icv bolus of RF9 (20 nmol) on LH and FSH levels in adult male rats supplemented (or not) with high levels of T are shown. In addition to time-course data, integrated hormonal responses to RF9 during the 120 min are presented as AUC; *crossed bars* represent estimated basal secretion in each group. B, Similar analyses are shown from adult female rats supplemented (or not) with high doses of E2. a, $P \leq 0.01$ *vs.* corresponding values in animals without sex steroid supplementation; **, $P \leq 0.01$ *vs.* corresponding preinjection values (*i.e.* before central RF9 administration) (ANOVA followed by Student-Newman-Keuls multiple-range test). In histograms, numeric values represent relative fold increases over corresponding basal levels in each group.

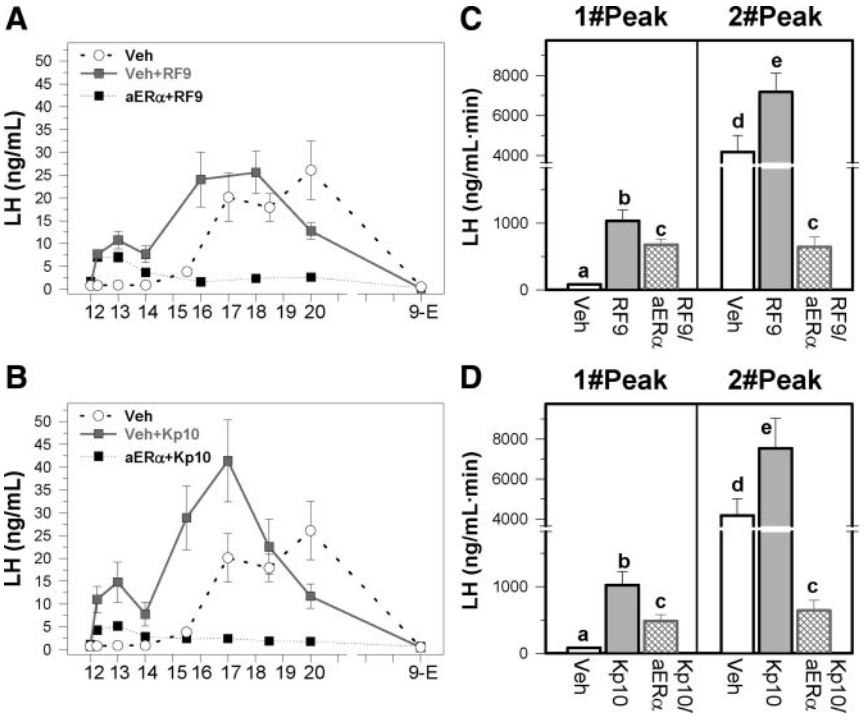

FIG. 7. Effects of RF9, Kp-10, or their coadministration after ERα blockade in cycling female rats. A, Effects of a single icv injection of RF9 (20 nmol) on circulating LH levels in female rats at proestrus, pretreated with effective doses of an ERα antagonist. For comparative purposes, the effects of RF9 on serum LH levels in proestrus females not treated with the ERα antagonist are presented. B, Analyses of LH responses to icv injection of Kp-10 (1 nmol) in the same experimental setting. C and D, To ease quantitative comparison of the hormonal data, in addition to time-course values, integrated hormonal responses to the different treatments were calculated as AUC, as described in *Materials and Methods*. Groups with *different superscript letters* are statistically different (ANOVA followed by Student-Newman-Keuls multiple-range test).

plored in recent years, and novel neuroendocrine regulators have been exposed. These include not only kisspeptins, as prominent stimulatory signals for GnRH neurons, and thereby very potent elicitors of gonadotropin secretion (5), but also the putative inhibitory factor RFRP as the mammalian ortholog of GnIH (13, 18, 38). Indeed, the fact that both kisspeptins and RFRP/GnIH belong to the superfamily of RFa peptides has led to the appealing hypothesis that these related neuropeptides might be the major driving forces for the reciprocal regulation of GnRH/gonadotropin secretion (26, 38). Yet, although the dominant roles of kisspeptins in the control of the gonadotropic axis are now undisputed (5), strong controversy persists on the actual physiological relevance and major mechanisms and sites of action of RFRP in the regulation of pituitary gonadotropins (13). This debate may stem, at least partially, from the use of experimental approaches based mostly on the testing of the effects of exogenous agonists as a means to provide evidence for its biological functions *in vivo*. In fact, most of the literature on this topic has been based on protocols of central or peripheral administration of rather high doses of RFRP-3 and assessment of hormonal responses and/or markers of neuronal activation. In contrast, to our knowledge, only one study has addressed the consequences of inhibition of endogenous RFRP synthesis, by means of central infusion of antisense oligonucleotides, which revealed a modest elevation of LH levels in antisense oligonucleotide-treated rats (28). Yet, in that particular study, a protocol of chronic infusion (2 wk) was selectively applied to pubertal male rats. In this context, the use of pharmacological antagonists might prove instrumental for a better dissection of the array of acute regulatory actions of RFRP in different functional states of the gonadotropic axis.

Based on previous reports on its properties as potent and selective antagonist of RFRPR and NPFFR (12), we undertook a thorough characterization of the effects of RF9 on gonadotropin secretion in rats and mice *in vivo*, at doses previously used for the validation of the antagonistic activity of this compound, mostly in rat models of nociception and opioid tolerance (12, 31, 32). In keeping with its predicted role as mediator of the inhibitory actions of RFRPs on the gonadotropic axis, blockade of RFRPR by central administration of RF9 resulted in very robust LH and, to a lesser extent, FSH secretory responses in rats and mice. Although the nature of the effect was not unpredicted, the large magnitude of such responses was somewhat surprising, given the modest amplitude of the inhibitory responses to RFRP-3 reported in most of the literature (13) and confirmed also in our pharmacological testing (Pineda, R., M. Blomenröhr, P. I. van Noort, and M. Tena-Sempere, in preparation). In any event, the repeated observation of such potent stimulatory actions in a large number of independent experiments conducted in two different rodent species, in both sexes, and against different functional states of the gonadotropic axis, fully support that this is a genuine phenomenon, whose physiological and pharmacological implications are yet to be fully elucidated.

In terms of physiology, the fact that central blockade of RFRPRs resulted in clear-cut elevations of circulating LH and FSH levels is compatible with a relevant role of RFRP-3 in the hypothalamic control of the gonadotropic axis, as indirectly suggested by a number of pharmacological studies (13). Indeed, the strong secretory responses

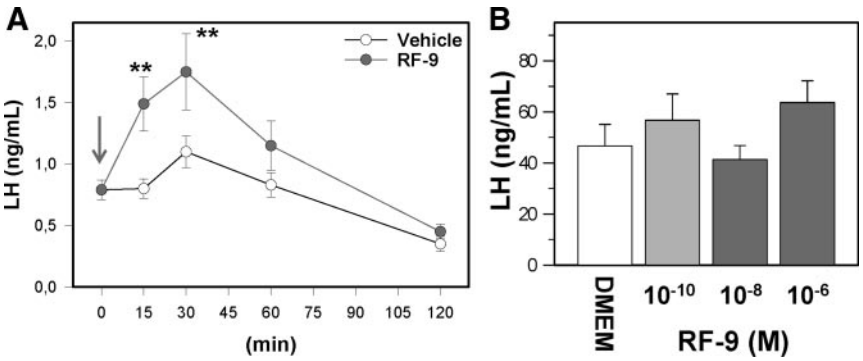

FIG. 8. Systemic and pituitary effects of RF9 on gonadotropin secretion in male rats. A, Effects of a single ip bolus of RF9 (0.1 mg/kg body weight) on LH levels in adult male rats are shown. B, LH concentrations in the incubation media from pituitaries exposed the increasing concentrations of RF9. **, $P \leq 0.01$ *vs.* corresponding control group (ANOVA followed by Student-Newman-Keuls multiple-range test).

detected after central injection of RF9 might be indicative of a constitutive RFRP inhibitory tone, which may tonically restrain the secretion of gonadotropins. In such conditions, the effects of an antagonist, which would block the endogenous RFRP tone, might be more readily detectable than the consequences of its augmentation by means of exogenous administration of RFRP-3. In addition, the observations that RF9 effects were less pronounced after peripheral injection and that no LH and FSH responses were detected directly at the pituitary strongly suggest a primary site of action of RFRPs at central levels (likely at the hypothalamus) in the control of the gonadotropic axis. Admittedly, however, the fact that RF9 also antagonizes NPFFRs does not allow for unequivocal interpretation of our hormonal data, because at least part of the observed changes in gonadotropin levels after RF9 administration may derive from blockade of NPFF actions. This seems very unlikely because this neuropeptide does not appear to be involved in the control of gonadotropin secretion (28) (Pineda, R., M. Blomenröhr, P. I. van Noort, and M. Tena-Sempere, in preparation). Yet, NPFF has been shown to display certain anti-opioid properties; endogenous opioids being well-known inhibitors of gonadotropin secretion (39–41). In addition, although the selectivity of RF9 for RFRPR and NPFFR has been previously documented (12), and the doses used herein are similar to those reported in the literature (12), we cannot rule out the possibility that part of the effects of RF9 may stem from activation/inactivation of other, as yet unknown, neuropeptide systems. On this point, however, it is important to stress that a previous study failed to demonstrate any significant binding of RF9 to several G protein-coupled receptors, including the kisspeptin receptor (GPR54) as well as other RFa peptide receptors (GPR10 and GPR103) at doses up to 10 μM (12), thus suggesting specificity of action. In this context, testing of the effects of coadministration of optimal doses of RF9 and RFRP3 as well as analyses of binding of this antagonist to relevant hypothalamic regions, such as the preoptic area where most of GnRH neurons are located, might prove helpful to conclusively define the mechanisms underlying the very potent gonadotropin-releasing effects of RF9.

Although central injection of RF9 evoked unambiguous LH and FSH responses in male and female rats, clear differences were detected, in terms of threshold doses and relative magnitude, between both gonadotropins. Thus, in male rats, significant LH responses to RF9 were detected at doses of 1 nmol icv, with a maximal 8-fold increase over basal levels at doses of 20 nmol. In contrast, elevation of FSH levels was observed only after icv injection of maximal doses of RF9 (20 nmol), which accounted for a 65% increase over corresponding basal concentrations (see Fig. 3). This differential pattern of response (*i.e.* lower threshold doses and higher relative increases for LH than for FSH) is analogous to those reported previously for other potent gonadotropin secretagogues, such as kisspeptins (5, 6), and is congruent with the respective modes of secretion of each gonadotropin (5). Such profiles are also compatible with a GnRH-mediated effect of RF9, a contention that is also supported by lack of direct pituitary effects of RF9 and the observation of greater gonadotropin responses in cycling female rats at estrus than at diestrus, in keeping with previous data on changes in kisspeptin responsiveness across the cycle (5, 6).

Given the potent gonadotropin-releasing effects of RF9 and the proposed interplay between RFRP and other major regulators of the gonadotropic axis, such as kisspeptins and sex steroids (18, 26, 38), specific studies were implemented to evaluate such potential interactions. Our analyses revealed that 1) the effects of RF9 in terms of gonadotropin secretion were at the very least similar (if not higher; see Fig. 5) to those of Kp-10, at a range of doses of 0.1–1 nmol icv, and 2) coadministration of Kp-10 and RF9 potentiated the stimulatory effects of each compound alone. These observations stress the relative potency of RF9 and are compatible with a reciprocal interaction between RFRP and kisspeptin in the dynamic control of gonadotropin secretion (26, 38). In addition, the stimulatory effects of RF9 were preserved (and even augmented in terms of relative responses) in models of negative feedback by sex steroids, observations that are in keeping with the proposed role of RFRPs in mediating part of the negative feedback regulation of gonadal steroids on the gonadotropic axis (18). Intriguingly, icv injection of RF9 ad-

vanced the occurrence of the endogenous ovulatory surge of gonadotropins, whereas the releasing effects of RF9 were partially blunted after blockade of ERα signaling during the preovulatory period. Whether the latter is related to a potential decrease in the endogenous RFRP tone during this stage of the cycle, as suggested previously (25), remains to be elucidated.

In addition to potential physiological relevance, our present observations have obvious pharmacological implications, on at least two fronts. First, RF9 has been proposed as the leading compound for the generation of drugs with capacity to interfere with opioid-induced tolerance and hyperalgesia (12, 31, 32). We demonstrate herein that at the very same doses of RF9 (icv and systemic) used previously in pharmacological studies in rat models of nociception (12), the antagonist is capable of evoking potent LH and FSH secretory responses, with the predicted downstream activation of the gonadal axis, a side effect whose impact in chronic pain management needs to be carefully considered. Second, the secretory actions of RF9 in terms of gonadotropin secretion reported here make this compound a suitable target in the generation of strategies for the hormonal manipulation of the reproductive axis, an approach whose pharmacological characteristics (*e.g.* in terms of desensitization, routes of administration, *etc.*) and potential therapeutic indications merit further investigation.

In conclusion, we provide herein the first demonstration that RF9, a selective and potent antagonist of RFRPR and NPFFR, previously proposed as potential drug treatment for improving the efficacy of opioids in the management of chronic pain, possesses strong gonadotropin secretory actions *in vivo*. These observations not only are of physiological interest, in terms of better characterization of the biological roles of RFRPs, as putative counterparts of GnIH in mammals, but also have potential pharmacological implications, which warrant specific analyses.

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