Hypophysiotropic role of RFamide-related peptide-3 in the inhibition of LH secretion in female rats

Masahiro Murakami, Toshiya Matsuzaki, Takeshi Iwasa, Toshiyuki Yasui, Minoru Irahara, Tomohiro Osugi[1] and **Kazuyoshi Tsutsui**[1]

Department of Obstetrics and Gynecology, The University of Tokushima Graduate School, Institute of Health Biosciences, 3-18-15 Kuramoto-Cho, Tokushima 770-8503, Japan

[1]Department of Biology, Waseda University, 2-2 Wakamatsu-Cho, Shinjuku-ku, Tokyo 162-8480, Japan

(Correspondence should be addressed to T Matsuzaki; Email: mat@clin.med.tokushima-u.ac.jp)

Abstract

Gonadotropin-inhibitory hormone (GnIH), a newly discovered hypothalamic RFamide peptide, inhibits reproductive activity by decreasing gonadotropin synthesis and release in birds. The gene of the mammalian RFamide-related peptides (RFRP) is orthologous to the *GnIH* gene. This *Rfrp* gene gives rise to the two biologically active peptides RFRP-1 (NPSF) and RFRP-3 (NPVF), and i.c.v. injections of RFRP-3 suppress LH secretion in several mammalian species. In this study, we show whether RFRP-3 affects LH secretion at the pituitary level and/or via the release of GnRH at the hypothalamus in mammals. To investigate the suppressive effects of RFRP-3 on the mean level of LH secretion and the frequency of pulsatile LH secretion *in vivo*, ovariectomized (OVX) mature rats were administered RFRP-3 using either i.c.v. or i.v. injections. Furthermore, the effect of RFRP-3 on LH secretion was also investigated using cultured female rat pituitary cells. With i.v. administrations, RFRP-3 significantly reduced plasma LH concentrations when compared with the physiological saline group. However, after i.c.v. RFRP-3 injections, neither the mean level of LH concentrations nor the frequency of the pulsatile LH secretion was affected. When using cultured pituitary cells, in the absence of GnRH, the suppressive effect of RFRP-3 on LH secretion was not clear, but when GnRH was present, RFRP-3 significantly suppressed LH secretion. These results suggest that RFRP-3 does not affect LH secretion via the release of GnRH, and that RFRP-3 directly acts upon the pituitary to suppress GnRH-stimulated LH secretion in female rats.

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Introduction

The hypothalamic–pituitary–gonadal axis is regulated by the facilitative effect of gonadotropin-releasing hormone (GnRH) in the hypothalamus of mammals and other vertebrates. In recent years, a novel avian hypothalamic neuropeptide-inhibiting gonadotropin release was discovered in quail and was designated gonadotropin-inhibitory hormone (GnIH; Tsutsui *et al.* 2000). GnIH is also effective in inhibiting gonadotropin release *in vitro* and *in vivo* in several avian species (Tsutsui *et al.* 2000, Ciccone *et al.* 2004, Osugi *et al.* 2004, Ubuka *et al.* 2006). In birds, cell bodies and terminals of GnIH neurons are localized in the paraventricular nucleus (PVN) and median eminence (ME) respectively (Tsutsui *et al.* 2000, Bentley *et al.* 2003, Ubuka *et al.* 2003, Ukena *et al.* 2003). GnIH acts directly on the pituitary via a novel G-protein-coupled receptor for GnIH to inhibit not only gonadotropin release but also its synthesis (Ciccone *et al.* 2004, Yin *et al.* 2005, Ubuka *et al.* 2006). Furthermore, GnIH inhibits gonadal development and maintenance by inhibiting gonadotropin release and synthesis in birds (Tsutsui *et al.* 2006, 2007, Ubuka *et al.* 2006).

The GnIH precursor encodes one GnIH and two GnIH-related peptides (GnIH-RP-1 and GnIH-RP-2) that include Leu-Pro-Xaa-Arg-Phe-NH_2 (Xaa = Leu or Gln) at their C-termini in birds (Satake *et al.* 2001, Osugi *et al.* 2004). Based on gene database searches, cDNAs that encode GnIH homologous peptides containing a C-terminal LPXRFamide motif have been detected in mammalian brains (Hinuma *et al.* 2000). Mammalian cDNAs encode the two biological active GnIH homologous peptides, i.e., RFamide-related peptide (RFRP)-1 and RFRP-3 whose alias are neuropeptide SF (NPSF) and neuropeptide VF (NPVF) respectively which are encoded by neuropeptide VF precursor (NPVF) gene (Hinuma *et al.* 2000, Liu *et al.* 2001). Up until now, the mammalian GnIH homologs, RFRP-1 and RFRP-3, have been identified in the bovine (both RFRP-1 and RFRP-3) and rat (RFRP-3 only) brains (Fukusumi *et al.* 2001, Ukena *et al.* 2003, Yoshida *et al.* 2003). I.c.v. administration of the deduced human RFRP-1 increased prolactin (PRL) release in the rat (Hinuma *et al.* 2000). By contrast, i.c.v. injections of RFRP-3 reduced plasma levels of luteinizing hormone (LH) in male rats (Johnson *et al.* 2007). When injected i.c.v. or

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intraperitoneally (i.p.), GnIH also reduced plasma LH levels in ovariectomized (OVX) Syrian hamsters (Kriegsfeld *et al.* 2006). GnIH and RFRP-3 are therefore considered to be functional homologs. In addition, both GnIH and RFRP-3 facilitate food intake in chicks (Tachibana *et al.* 2005) and male rats (Johnson *et al.* 2007) respectively.

Recent studies have shown that GnIH neurons project not only to the ME but also to GnRH neurons in birds (Bentley *et al.* 2003, Ubuka *et al.* 2008). GnIH receptor is also expressed in GnRH neurons (Bentley *et al.* 2006, Ubuka *et al.* 2008). Therefore, GnIH may act at the level of the hypothalamus to inhibit gonadotropin release and synthesis as well as acting at the level of the pituitary in birds. On the other hand, RFRPs are mainly expressed in the dorsomedial hypothalamic nucleus/PVN in mammals (Hinuma *et al.* 2000, Fukusumi *et al.* 2001, Ukena & Tsutsui 2001, Yano *et al.* 2003, Kriegsfeld *et al.* 2006, Johnson *et al.* 2007). The RFRPs-immunoreactive fibers are broadly distributed in several brain areas, such as the bed nucleus of the stria terminalis, medial preoptic area, medial and lateral septal areas, paraventricular thalamic nucleus, etc. (Ukena & Tsutsui 2001, Yano *et al.* 2003, Kriegsfeld *et al.* 2006, Johnson *et al.* 2007). According to several studies in rodents (Kriegsfeld *et al.* 2006, Johnson *et al.* 2007), some of the RFRPs-immunoreactive fibers project to the outer layer of the ME, while other RFRPs-immunoreactive fibers contact a large percentage of the GnRH neurons in the hypothalamus. The receptor for the RFRPs, which is referred to as RFRP-R (also found as OT7T022 or NPFF1), is a G-protein-coupled receptor that is widely expressed in several brain areas including the hypothalamus and the pituitary in rats (Hinuma *et al.* 2000). The reduction of LH secretion following i.c.v. injections of RFRP-3 and the distribution of RFRPs-immunoreactive fibers in the hypothalamus suggest that RFRP-3 may affect LH secretion via GnRH release in rodents. By contrast, the localization of RFRP-R suggests that RFRP-3 may act on both the pituitary and GnRH neurons in the hypothalamus to inhibit LH secretion. Up until now, however, no information on the direct action of RFRP-3 on the pituitary as a hypophysiotropic hormone has been available in rodents or in other mammalian species. Thus, the mode of action of RFRP-3 on the regulation of LH secretion has not been clarified in mammals. With these findings as background, the present *in vitro* and *in vivo* studies with OVX rats were conducted to determine whether RFRP-3, which is a mammalian GnIH homolog, affects LH secretion at the pituitary level and/or via GnRH release.

Materials and Methods

Animals

Adult Wistar rats (Charles River Japan Inc., Yokohama, Japan) weighing 200–220 g were used in all experiments. They were housed in a temperature-controlled room (24 °C) under a daily photoperiod of 14 h light:10 h darkness (lights on at 0700 h) and given food and tap water *ad libitum*. All animal experiments were conducted in accordance with the ethical standards of the institutional Animal Care and Use Committee of the University of Tokushima.

Experiment 1: effect of an i.v. injection of RFRP-3 on plasma LH levels

All rats were bilaterally OVX at 8 weeks of age. A silastic tube (1·0 mm o.d., 0·5 mm i.d., Kaneka Medics. Tokyo, Japan) was inserted into the right atrium via the external jugular vein and was exteriorized at the back of the head. The tube was rinsed with heparinized saline (10 000 U/l). On the following day, the intra-atrial cannula was rinsed and connected to a long polyethylene tube containing heparinized saline. A steel pin was inserted into the open end of this tube, which was led outside the cage to permit rapid blood sampling without handling the rats. All surgical procedures were carried out under anesthesia with pentobarbital sodium (40 mg/kg body weight, i.p.), as has been reported previously (Tamura *et al.* 1999). Six rats in the RFRP-3 group were injected with 1 μg of RPRP-3 i.v., and six rats in the control group were injected with saline i.v. The injection volume was 5 μl in both groups. Blood (200 μl) was collected from each rat at 0, 30, 60, and 120 min after the i.v. injection of RFRP-3 or saline. Blood samples were centrifuged and plasma was stored at −40 °C until LH concentrations were measured.

Experiment 2-1: effect of an i.c.v. injection of RPRP-3 on pulsatile LH secretion

At 10–14 days after OVX, an i.c.v. cannula was implanted into the third cerebroventricle (3V) using stereotaxic coordinates published in the atlas of Paxinos & Watson (1986). A guide cannula comprising a 23 gage stainless steel tube (20 mm long, 0·64 mm o.d., 0·39 mm i.d.) was implanted, and a sterile 29 gage stainless steel obturator with a polyethylene cap (20 mm×0·33 mm o.d.) was inserted into the guide cannula to ensure that the cannula remained patent. One week after implantation, rats were used for the experiments.

A silastic tube was inserted into the right atrium via the external jugular vein and was exteriorized at the back of the head. The tube was rinsed with heparinized saline. On the day after atrial cannulation, RFRP-3 or saline was injected into the 3V using a Hamilton microsyringe. Rats were divided into two experimental groups. Four rats in the RFRP-3 group were injected with 500 ng of RFRP-3 into the 3V, while four rats in the control group were injected with saline into the 3V. The injection volume was 5 μl in each group. Blood samples of 200 μl were collected through the i.v. cannula at 6-min intervals for 120 min under conditions that allowed for unrestricted movement of the rats. After each sampling, blood was replaced with an equal volume of heparinized saline (10 IU/ml). All surgical procedures were carried out under anesthesia with pentobarbital sodium (40 mg/kg body weight,

i.p). Samples were centrifuged and plasma was stored at −40 °C until LH concentrations were measured.

Experiment 2-2: effect of an i.c.v. injection of RFRP-3 on food intake

We tried to prove the activity of RFRP-3 and the validity of our i.c.v. injection technique by demonstrating the orexinergic effect of RFRP-3 using male rats with just the same method as Johnson *et al.* (2007) reported recently. An i.c.v. cannula was implanted into the 3V of 10-week-old male rats ($n=12$). Six rats in the RFRP-3 group received an i.c.v. injection of RFRP-3 (500 ng/5 μl) and six rats in the control group received saline (5 μl) at 0900 h during the photophase. To estimate food intake, we measured the remaining food weight 2 h later. This dose of RFRP-3 has previously been shown to stimulate food intake in male rats (Johnson *et al.* 2007). Rats were given food and tap water *ad libitum*. After injection, rats were returned to their home cages with a preweighed amount of rat food. To estimate food intake, we measured the remaining food weight 2 h later. The food was placed on the dish at the corner of the cage and little spilled food was observed during the experiment.

Experiment 3: effect of RFRP-3 on LH secretion in pituitary cell cultures

Ten-week-old female rats were decapitated, with their pituitaries immediately collected. Pituitaries were cut into small pieces and washed in Dulbecco's modified Eagle's medium (DMEM; Nissui Co., Tokyo, Japan). These pieces were subjected to enzymatic dispersion for 40 min at 37 °C using 0·25% trypsin, followed by dissociation performed by pipetting 0·2% pancreatin at 37 °C for 1 min, as has been previously reported (Kanematsu *et al.* 1991, Tezuka *et al.* 2002). Pituitary cells were seeded in DMEM containing 10% fetal bovine serum, and then plated on 24-well culture dishes (Falcon Plastics, Los Angeles, CA, USA) at a density of 10^6 viable cells/well. After preincubation for 24 h, the medium was changed, followed by incubation for 24 h in culture medium alone as control, with 10^{-9} M of GnRH (Wako Pure Chemical Industries, Ltd, Osaka, Japan) and with 10^{-8} M of RFRP-3. In another experiment, cells were incubated in culture medium with 10^{-9} M of GnRH (group A) or culture medium containing various concentrations of RFRP-3 (10^{-16}, 10^{-14}, and 10^{-12} M; groups B, C, and D respectively) with 10^{-9} M of GnRH. Cell cultures were maintained at 37 °C under a mixture of 95% air and 5% CO_2 at 100% humidity. After culturing for 24 h, media were collected and subjected to RIA for LH.

Hormone assay

Serum LH concentrations were measured by RIA with rat LH standards (Amersham Pharmacia Biotech). Sensitivity of the assay was 0·2 ng/ml. The inter- and intra-assay coefficients of variation (CV) were 6·6 and 6·5% respectively.

Statistical analysis

LH pulses were defined and identified using established criteria, as described by Gallo (1981) and DePaolo *et al.* (1987). Concretely, CV was calculated from LH concentrations on the ascending and descending phases of a suspected pulse. A pulse was assumed to have occurred if the CV was greater than twice the CV of the LH RIA that was determined from solutions of the LH standards that corresponded to the mean LH levels in the suspected pulse. The pulse amplitude denotes the difference between the peak and the baseline. The mean LH concentration, the pulse frequency (number of pulses during a period of 2 h), and the mean pulse amplitude were calculated for each animal. This method has been shown to reveal differences among groups in the parameters of pulsatile hormone release that are similar to differences obtained using the cluster algorithm (Culler 1990). Data were analyzed using one-way ANOVA followed by Fisher's protected least significance difference test in experiment 2-1. Mann–Whitney *U* test and Wilcoxon Rank test were used for comparisons between groups in experiment 1, 2-2, and 3. All statistical analysis was performed using StatView for Macintosh version 5.0 (SAS Institute Inc., Cary, NC, USA). Differences were considered to be statistically significant at $P<0·05$. All results are presented as the mean ± S.E.M., with $n=4$–6 samples per group as indicated.

Results

Experiment 1: effect of an i.v. injection of RPRP-3 on plasma LH levels

After an i.v. administration of RPRP-3, serum LH levels decreased gradually for 120 min, at which time (4·15 ± 0·46 ng/ml, mean ± S.E.M.; $n=4$) a significant difference was seen when compared with the levels observed at 0 min (6·31 ± 0·46, $n=4$; $P=0·028$; Fig. 1). In the control group, serum LH levels did not change for 120 min (7·42 ± 1·38, $n=4$) after saline injection and significant differences were seen when compared with the RFRP-3 group at 120 min ($P=0·018$, Fig. 1).

Experiment 2-1: effect of an i.c.v. injection of RPRP-3 on pulsatile LH secretion

Representative LH secretion profiles of two rats in each group are depicted in Fig. 2. Rats in the RFRP-3 group showed frequent LH secretion pulses when compared with the rats in the control group. Effects of an i.c.v. injection of RFRP-3 on the mean LH concentration (control versus RFRP-3: 8·34 ± 0·95 ng/ml, mean ± S.E.M., $n=4$ vs 7·41 ± 0·40, $n=4$), pulse frequency (4·60 ± 0·25/120 min, $n=4$ vs 4·20 ± 0·20, $n=4$), and pulse amplitude (5·35 ±

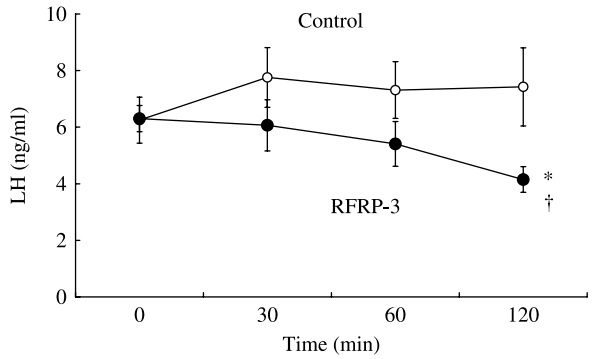

Figure 1 Effect of an i.v. injection of RFRP-3 on serum LH concentrations in OVX rats. Values represent the mean±S.E.M. (n=6). *P=0·028 versus RFRP-3 at 0 min; †P=0·018 versus control at 120 min.

0·53 ng/ml, n=4 vs 5·29±0·44, n=4) are summarized in Fig. 3. There were no significant differences in these parameters between the two groups, suggesting that there was no significant suppressive effect of the i.c.v. administration of RFRP-3 on pulsatile LH secretion.

Experiment 2-2: effect of an i.c.v. injection of RPRP-3 on food intake

Food intake data after an i.c.v. injection of RFRP-3 at 500 ng (same dose as in Experiment 2-1) are shown in Fig. 4. A significantly higher food intake during the photophase when compared with the control group (0·51±0·32 g/2 h, mean±S.E.M.; n=6) was seen after an i.c.v. injection of RFRP-3 (1·67±0·52, n=6) in adult male rats (P=0·028, Fig. 4).

Experiment 3: effect of RFRP-3 on LH secretion on pituitary cell cultures

When pituitary cells were cultured under GnRH-deficient conditions, RFRP-3 administration did not suppress LH secretion, whereas GnRH administration significantly stimulated LH secretion (P=0·033 versus control, P=0·049 versus RFRP-3; Fig. 5). By contrast, the secretion of LH (group D: 5·08±0·45 ng/ml, mean±S.E.M.; n=4) from the pituitary cells in the medium containing RFRP-3 (10^{-12} M) and GnRH (10^{-9} M) was significantly suppressed when compared with only GnRH (10^{-9} M; group A: 7·58±0·49, n=4), RFRP-3 (10^{-16} M) and GnRH (10^{-9} M; group B: 8·17±0·45, n=4), and RFRP-3 (10^{-14} M) and GnRH (10^{-9} M; group C: 6·83±0·38, n=4; P=0·033 versus group A, P=0·020 versus group B, P=0·040 versus group C; Fig. 6). At lower concentrations of RFRP-3 than 10^{-12} M, there was either no effect or a significant reduction in the secretion of LH compared with only GnRH (10^{-9} M).

Discussion

GnIH, a newly discovered hypothalamic RFamide peptide, inhibits gonadotropin release *in vitro* and *in vivo* in birds (Tsutsui *et al*. 2000, Ciccone *et al*. 2004, Osugi *et al*. 2004, Ubuka *et al*. 2006). Because GnIH neurons project to the ME and GnRH neurons (Tsutsui *et al*. 2000, Bentley *et al*. 2003, Ubuka *et al*. 2003, 2008, Ukena *et al*. 2003) and GnIH receptor is expressed in the pituitary (Yin *et al*. 2005) and GnRH neurons (Bentley *et al*. 2006, Ubuka *et al*. 2008), GnIH may act not only at the pituitary level but also at the hypothalamic level to inhibit gonadotropin secretion in birds. RFRP-3, a mammalian

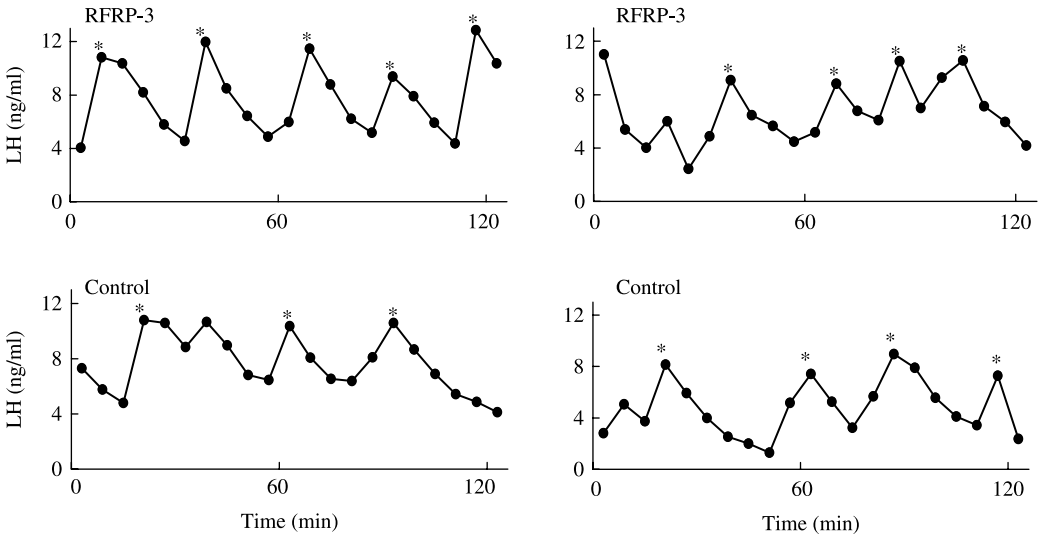

Figure 2 Effect of an i.c.v. injection of RFRP-3 on pulsatile secretion of LH in OVX rats. Representative profiles of two animals in the control and RFRP-3 groups are shown. RFRP-3 (500 ng/ml) was injected at 0 min and blood samples were taken at 6-min intervals for 120 min. Asterisks denote LH secretion pulses.

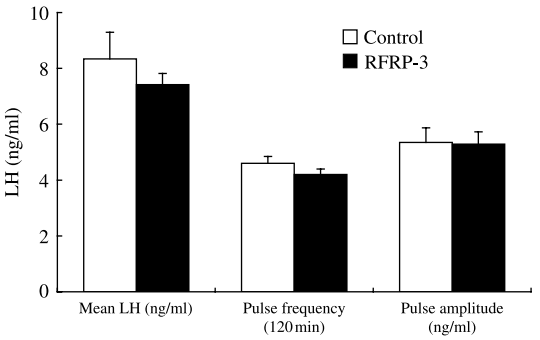

Figure 3 Summary of the effects of an i.c.v. injection of RFRP-3 on the parameters of the pulsatile secretion of LH, i.e., the frequency and amplitude of the LH pulses and mean LH concentration, in OVX rats. Values represent the mean±S.E.M. (n=4).

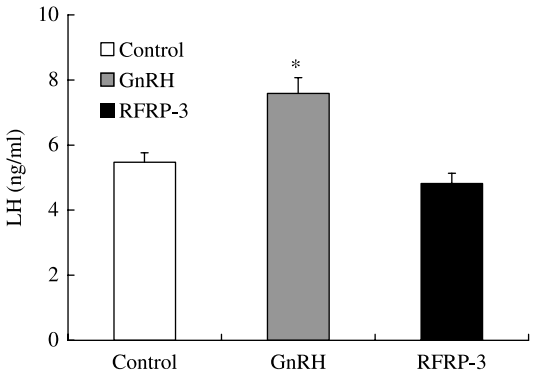

Figure 5 Effect of RFRP-3 administration on the 24-h release of LH from cultured pituitary cells. Values represent the mean±S.E.M. (n=4). *P=0·033 versus control, *P=0·049 versus RFRP-3.

GnIH homolog, has been identified in the rat and bovine (Fukusumi *et al.* 2001, Ukena *et al.* 2003, Yoshida *et al.* 2003). Both GnIH and RFRP-3 share a common C-terminal LPXRFamide motif and are functionally the same (for review, see Tsutsui & Ukena 2006). Also, both RFRP-3 (Johnson *et al.* 2007) and GnIH (Kriegsfeld *et al.* 2006) have been shown to suppress gonadotropin release *in vivo* in rodents. Therefore, it is considered that RFRP-3, which is a mammalian GnIH homolog, might be an important factor in inhibiting gonadotropin release in mammals, similar to the action of GnIH in birds. To clarify the mode of action of RFRP-3 on the regulation of LH secretion, in this study we performed *in vitro* and *in vivo* experiments using mature female rats in order to investigate whether the suppressive effect of RFRP-3 on LH secretion occurs at the level of the hypothalamus and/or pituitary.

In this study, we first found that RFRP-3 reduced plasma LH levels in OVX rats after an i.v. injection of RFRP-3. Subsequently, we studied the effect of RFRP-3 on the pulsatile secretion of GnRH by analyzing the fluctuation of the plasma LH levels in OVX rats. Our results indicated that an i.c.v. injection of RFRP-3 in OVX rats did not suppress the plasma LH levels or affect the frequency of the pulsatile LH secretion. In addition, i.c.v. injections of higher doses of RFRP-3 (1 and 10 μg; data not shown) and avian GnIH (1 and 10 μg; data not shown) did not have any effect on pulsatile LH secretion, suggesting that there was no significant impact on GnRH secretion by RFRP-3 in OVX rats. Furthermore, when using cultured pituitary cells, RFRP-3 significantly suppressed LH secretion in the presence of GnRH. We confirmed the activity of the RFRP-3 preparations used in this study and the validity of our experiments by demonstrating that there was an increased food intake after an i.c.v. injection of RFRP-3 in a male rat.

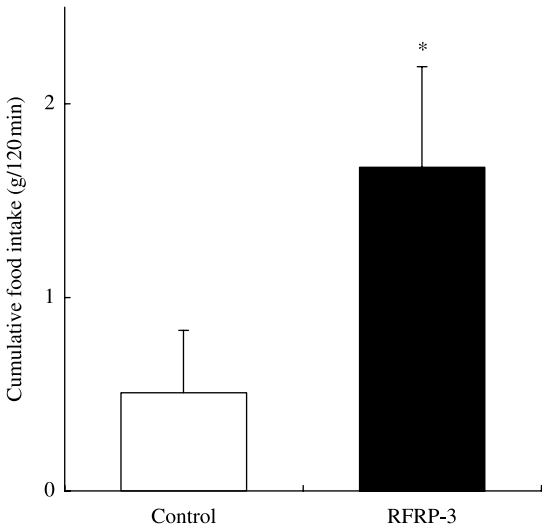

Figure 4 Effect of an i.c.v. injection of RFRP-3 on food intake during the photophase in adult male rats. Values represent the mean± S.E.M. (n=6). *P=0·028 versus control.

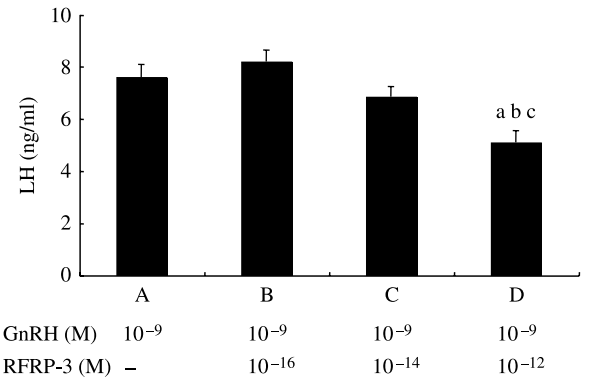

Figure 6 The dose–response effects of RFRP-3 administration on the 24-h release of LH from cultured cells in the presence of GnRH. Addition of 10^{-12} M RFRP-3 (group D) showed significant suppressive effect in GnRH (10^{-9} M)-stimulated LH secretion compared with other groups with no RFRP-3 (group A) or with lower RFRP-3 concentrations (groups B and C). Values represent the mean±S.E.M. (n=4). a, P=0·033 versus group A; b, P=0·020 versus group B; c, P=0·040 versus group C.

The results of the current study concur with those reported by Johnson *et al.* (2007) who used the same method that we used. Although in the past most of the experimental focus has been on the central action of RFRP-3 and the inhibition of GnRH function in mammals, when taken together, the present results indicate that RFRP-3 acts as a hypophysiotropic hormone on the pituitary to inhibit LH secretion. Thus, the results suggest that RFRP-3, which is one of the mammalian GnIH homologs, directly acts on the pituitary as a hypophysiotropic factor in order to suppress GnRH-stimulated LH secretion in OVX rats. This suppressive action of RFRP-3 on LH secretion may not be mediated by the inhibition of GnRH.

As we have reported previously, i.c.v. injections of GnRH inhibiting factors such as hypocretin (HCRT; also known as orexin) and 2-buten-4-olide suppressed plasma LH levels immediately after injection, with the suppression being sustained for at least 1 h (Saito *et al.* 1993, Kaji *et al.* 1998, Tamura *et al.* 1999, Irahara *et al.* 2001, Iwasa *et al.* 2007). Kriegsfeld *et al.* (2006) deduced that RFRP-3 suppresses LH secretion at the hypothalamic level, as more than 40% of the GnRH neurons were projected from the GnIH-immunoreactive fibers. After an i.c.v. injection of avian GnIH, they found that there was an immediate suppression of the plasma LH levels in OVX hamsters. Based on the results of a study that examined the RFRP-3 neuronal projection and RFRP-3 i.c.v. injections, Johnson *et al.* (2007) deduced that RFRP-3 suppressed LH at the hypothalamic level in male rats. These findings were similar to the results reported by Kriegsfeld *et al.* (2006). In Kriegsfeld's study, 500 ng of avian GnIH was administered i.c.v. to OVX hamsters. Starting 5 min after the injection, they confirmed the presence of low LH levels. This differs from the results that we found in the present study. The reason for this may be due to the different species of animal that were used in the two studies. Moreover, based on gene database searches, RFRP-1, which is another mammalian GnIH homolog, has been suggested to exist in rats (Hinuma *et al.* 2000). While the rat RFRP-1 has yet to be identified, and, additionally, the differences in receptor affinity and GnRH intracellular transmission activation between RFRP-1 and RFRP-3 have yet to be clarified, it might be possible that RFRP-1 suppresses GnRH secretions. On the other hand, the reason for the differences between the data reported by Johnson *et al.* (2007) and the present study could be due to gonadectomy and gender differences. In OVX rats, endogenous GnRH secretion is excessive, and this could have masked the suppressive effect of RFRP-3 on GnRH secretion if its effect was not as strong as that found for the hypocretins (Tamura *et al.* 1999). Thus, our present findings suggest that RFRP-3 may not act at the level of the hypothalamus, as we could not detect any significant effect of RFRP-3 on the pulsatile secretion of GnRH in OVX rats.

On the other hand, we injected RFRP-3 i.v. in OVX rats and confirmed that RFRP-3 reduced plasma LH levels. Kriegsfeld *et al.* (2006) administered 600 ng of avian GnIH i.p. in OVX hamsters and demonstrated that by 30 min after the injections, there were suppressed LH levels. These results suggested that the suppressive effect of RFRP-3 on LH secretion occur at the level of the pituitary. Moreover, similar to that seen in birds (Tsutsui *et al.* 2000), we demonstrated for the first time the suppressive effect of RFRP-3 on LH secretion in the presence of GnRH when using pituitary cell cultures. The stimulating effect of GnRH (10^{-9} M) was almost diminished by RFRP-3 at 10^{-12} M, and this concentration of RFRP-3 was only 1/1000 when compared with that of GnRH added in the medium. In this way, RFRP-3 showed the strong suppressive effect of GnRH-stimulated LH secretion from cultured pituitary cells. By contrast, the suppressive effect of RFRP-3 on LH secretion was not seen in the absence of GnRH. These findings are in accordance with the classical report that gonadotropin secretion is maintained by the presence of GnRH stimulation (Clarke *et al.* 1983). However, to conclusively demonstrate that RFRP-3 is antagonistic to GnRH at the pituitary via the pituitary-portal system, further detailed analyses are needed.

In the classic study of Harris & Jacobsohn (1950, 1952), transplantation of the pituitary from the sella turcia to the renal capsule resulted in atrophy of the thyroid, the cortex of adrenal, and the gonad. Kanematsu & Sawyer (1973) showed elevated plasma PRL levels after the hypophyseal stalk was transected in the rat. These results showed that the neuroendocrine control of the pituitary hormones from the hypothalamus via the pituitary-portal system were dominantly controlled by stimulators, with the exception of PRL, which was regulated negatively by dopamine (Harris & Jacobsohn 1950, 1952, MacLeod 1969, Kanematsu & Sawyer 1973). Additionally, neuroendocrinological control of gonadotropin from the hypothalamus is maintained primarily via the stimulation associated with GnRH. We speculate that RFRP-3 has a complemental role in regulating gonadotropin secretion by antagonizing the facilitative control of GnRH at the pituitary level. Several factors have been reported to regulate gonadotropin secretion directly at the pituitary level. Stimulators include GnRH, leptin, activin B, and insulin-like growth factor I, whereas neuromedin U, inhibin A, and inhibin B are inhibitors (Gharib *et al.* 1990, Corrigan *et al.* 1991, Soldani *et al.* 1995, Besecke *et al.* 1996, Yu *et al.* 1997, Ogura *et al.* 2001, Tezuka *et al.* 2002, Gregory & Kaiser 2004, Fukue *et al.* 2006, Shimizu *et al.* 2008*a*,*b*). Thus, GnIH/RFRP-3 would be considered to be one of the inhibitors. Sources of these factors are quite varied and include the central nervous system. RFRP-3 itself is not expressed in the pituitary (Hinuma *et al.* 2000). However, it has been reported that sparse RFRP fibers extend into the external layer of the ME in the hamster (Kriegsfeld *et al.* 2006) and the rat (Johnson *et al.* 2007). Recent studies that have employed refined amplified immunohistochemical procedures have demonstrated a more pronounced innervation of the RFRP fibers in the ME in the hamster (Gibson, Humber, Jain, Williams, Zhas, Bentley, Tsutsui, Kriegsfeld, unpublished). In sheep, RFRP fibers have also been shown to project to the external zone of the ME

(Clarke, Sari, Qi, Smith, Parkington, Ubuka, Iqbal, Li, Tilbrook, Morgan, Pawson, Tsutsui, Millar, Bentley, unpublished). In addition to these findings, RFRP receptors are expressed in the pituitary of rodents (Hinuma *et al*. 2000) and humans (Ubuka, Smith, Sari, Parkington, Qi, Tilbrook, Morgan, Pawson, Osugi, Chowdhury, Minakata, Tsutsui, Clarke, Millar, Bentley, unpublished). These results confirm our present findings that RFRP-3, which is a mammalian GnIH homolog, directly acts on the pituitary to suppress the facilitative effect of GnRH on LH secretion in female rats. However, additional studies are required to draw a firm conclusion.

The presence of neuronal projections from the RFRP-3-immunoreactive cells into GnRH-immunoreactive cells strongly suggests that RFRP-3 somehow affects the GnRH neuron. GnRH secretion can be divided into the following two modes: one is the pulsatile secretion that regulates basic secretion of gonadotropin, while the other is the surge secretion that is responsible for the GnRH/LH surge that triggers ovulation. Each of these modes is controlled by different regulatory mechanisms. Since RFRP-3 did not affect the pulsatile secretion of GnRH in our study, this suggests that RFRP-3 may be involved in the regulation of the GnRH surge. The GnRH surge is generated by a positive feedback of estrogen that is released from the dominant follicle. Furthermore, several neuropeptides such as neuropeptide Y (NPY), HCRT, gamma aminobutyric acid (GABA), and kisspeptin/-metastin have been suggested to be involved in the GnRH surge (Adler & Crowley 1986, Kalra *et al*. 1988, Funabashi *et al*. 2002, Small *et al*. 2003, Kinoshita *et al*. 2005). Estrogen receptor-α is expressed by a large subset (39·41%) of RFRP-3 neurons and estrogen enhances the activity of the RFRP-3 neuron in female hamsters (Kriegsfeld *et al*. 2006). Therefore, the increased expression of RFRP-3 by estrogen might modulate the GnRH surge in conjunction with other factors. With regard to the effect of RFRP-3 on the GnRH neuron, additional studies are needed to clarify whether and how this effect occurs before we can draw the exact physiological role that RFRP-3 may have in gonadotropin secretion.

In conclusion this study indicated that RFRP-3, which is one of the mammalian GnIH homologs, directly acts on the pituitary to suppress the facilitative effect of GnRH on gonadotropin secretion in OVX rats, and that RFRP-3 may not act at the level of the hypothalamus, as we were unable to detect any significant effect of RFRP-3 on the pulsatile secretion of GnRH in OVX rats.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific works.

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