RESEARCH

Role of RFRP-3 in the development of cold stress-induced polycystic ovary phenotype in rats

V Squicciarini[1], R Riquelme[1], K Wilsterman[2], G E Bentley[2,3] and H E Lara[1]

[1]Center for Neurobiochemical Studies in Endocrine Diseases, Department of Biochemistry and Molecular Biology, Faculty of Chemistry and Pharmaceutical Sciences, Laboratory of Neurobiochemistry, Universidad de Chile, Santiago, Chile
[2]Department of Integrative Biology, UC Berkeley, Berkeley, California, USA
[3]Helen Wills Neuroscience Institute, UC Berkeley, Berkeley, California, USA

Correspondence should be addressed to H E Lara: hlara@ciq.uchile.cl

Abstract

RFamide-related peptide (RFRP-3) is a regulator of GnRH secretion from the brain, but it can also act in human ovary to influence steroidogenesis. We aimed to study the putative local role of RFRP-3 in the ovary and its potential participation in the development of a polycystic ovary phenotype induced by chronic sympathetic stress (cold stress). We used adult Sprague–Dawley rats divided into control and stressed groups. In both groups, we studied the effect of intraovarian exposure to RFRP-3 on follicular development and plasma ovarian steroid concentrations. We also tested the effect of RFRP-3 on ovarian steroid production *in vitro*. Chronic *in vivo* intraovarian exposure to RFRP-3 decreased basal testosterone concentrations and cold stress-induced progesterone production by the ovary. *In vitro*, RFRP-3 decreased hCG-induced ovarian progesterone and testosterone secretion. Immunohistochemistry and mRNA expression analysis showed a decrease in *Rfrp* and expression of its receptor in the ovary of stressed rats, a result which is in line with the increased testosterone levels found in stressed rats. *In vivo* application of RFRP-3 recovered the low levels of secondary and healthy antral follicles found in stressed rats. Taken together, our data indicate a previously unknown response of hypothalamic and ovarian RFRP-3 to chronic cold stress, influencing ovarian steroidogenesis and follicular dynamics. Thus, it is likely that RFRP-3 modulation in the ovary is a key component of development of the polycystic ovary phenotype.

Key Words

- stress
- polycystic ovary
- RFRP-3
- ovary

Journal of Endocrinology (2018) **239**, 81–91

Introduction

RFamide-related peptide (also known as gonadotropin-inhibitory hormone, GnIH) is a neuropeptide that is present in human brain and gonads and is known to act on human ovary (Tsutsui *et al.* 2007, Tsutsui 2009, Ubuka *et al.* 2009*b*, Singh *et al.* 2011*a*, Oishi *et al.* 2012). Functional evidence from humans and other species suggests that the peptide could be involved in pathologies that affect ovulation, follicular development and steroid secretion by the ovary (Ubuka *et al.* 2009*b*, Oishi *et al.* 2012, Bentley *et al.* 2017). One of these pathologies, polycystic ovary syndrome (PCOS), is the most common ovarian disease in reproductive age women (Lara *et al.* 1993). One proposed etiologic factor for PCOS is an increased secretion of gonadotropin-releasing hormone

http://joe.endocrinology-journals.org
https://doi.org/10.1530/JOE-18-0357

(GnRH) causing changes in ovarian function via pituitary gonadotropins (Chang 2007, Goodarzi *et al.* 2011). RFRP-3 inhibits gonadotropin release via its action on hypothalamic GnRH and on the anterior pituitary (Bentley *et al.* 2009, Ducret *et al.* 2009, Rizwan *et al.* 2014). Thus, there may be a role for hypothalamic RFRP-3 in the etiology of PCOS. However, in the present study, we focus on RFRP-3 and its receptor (GPR147 or NPFF1) within the rat ovary and investigate the potential that the gonadal RFRP system could act as a local regulator of ovarian function during the onset of PCOS (Singh *et al.* 2011*a*, Oishi *et al.* 2012).

Independently of neuroendocrine control, in humans and rats, an increase in sympathetic nervous activity is related to the onset of PCOS (Heider *et al.* 2001, Sverrisdottir *et al.* 2008). The autonomic response to cold exposure increases the secretion of norepinephrine (NE) from sympathetic nerve terminals (Benedict *et al.* 1979). Based on this observation, we have established a rat PCOS model, which involves chronic exposure to a cold sympathetic stress stimulus (Bhatnagar *et al.* 1995, Dorfman *et al.* 2003, Bernuci *et al.* 2008). Goldstein and Kopin (2008) have also demonstrated that this manipulation increases plasma NE without affecting the adrenal axis and thus corticosterone concentrations. We have independently demonstrated that our chronic cold stress protocol does not affect corticosterone plasma levels; thus, physiological changes occurring with this manipulation are the result of increased NE and not corticosterone (Dorfman *et al.* 2003). One of the key symptoms of PCOS is the excessive production of androgens. This arises partly as a result of excess production of luteinizing hormone (LH) from the pituitary gland (Azziz *et al.* 2006), but there might also be local regulation of androgen production from the ovary by ovarian RFRP-3, and dysfunction in ovarian RFRP-3 action could contribute to PCOS. Therefore, we examined the effect of exposure to sympathetic (cold) stress on PCOS in rats and investigated associated changes in the hypothalamic and ovarian RFRP systems.

Materials and methods

Animals and experimental design

Sprague–Dawley rats from our facilities, weighing 250–300 g were maintained at 20°C with a 12-h light and 12-h darkness cycle. Water and food were available *ad libitum*. The estrus cycling activity of the rats was monitored via daily vaginal smears observed under a light microscope as previously described (Fernandois *et al.* 2012). The number of cycles was estimated as the regular passage from proestrus (P) to estrus (E) followed by diestrus (D). All experimental procedures were approved by the Bioethics Committee of the Faculty of Chemistry and Pharmaceutical Sciences at the University of Chile (Protocol number: CBE2015-25) and complied with national guidelines (CONICYT Guide for the Care and Use of Laboratory Animals).

In vitro local steroid secretion studies

To study the capacity of RFRP-3 to modify local steroid secretion from the ovary, we used 20 adult rats (2-month-old) during proestrus. Rats were randomly divided into groups of five. Rats were killed by decapitation and both ovaries were removed through an anterior incision in the midline of the abdomen. Ovaries were halved (2 ovaries = 4 halves per animal), and each half was incubated for 3 h at 37°C in 1.0 mL of Krebs-bicarbonate-albumin (NaCl 118.6 Mm; KCl 4.7 mM; KH_2PO_4 1.2 mM; ascorbic acid 100 µg/mL; $NaHCO_3$ 0.15 M; $CaCl_2$ 25 mM; albumin 0.1 mg/mL; glucose 11.2 µg/mL) under 95% oxygen and 5% CO_2. For each rat, one half ovary was incubated in Krebs-bicarbonate buffer, and the second was incubated with additional 2.5 IU of hCG as a stimulus for steroidogenesis (Sigma Chemical Co.). The third half ovary was incubated in Krebs-bicarbonate buffer with 10 ng/mL RFRP-3 (NPFV, Phoenix Pharmaceuticals Inc., USA) and the fourth in buffer with the combination of both hCG and RFRP-3. After 3 h of incubation, the incubation media were collected to measure basal and induced secretion of testosterone, progesterone and estradiol by EIA kit, following the manufacturer's instructions. The catalog number of the test kits were 11-TESHU-E01, 11-ESTHU-E01 and 11-PROHU-E01 for testosterone, E2 and P4, respectively (Alpco Diagnostic, Windham, NH, USA). The concentration of RFRP-3 and hCG has been previously used to influence steroid secretion from immature and adult rodent ovaries (Barria *et al.* 1993, Singh *et al.* 2011*b*).

In vivo sympathetic stress and RFRP-3 administration studies

Twenty-four adult female rats were randomly assigned to either a non-stress group (control group) ($n = 12$) or stress group ($n = 12$). Each of them was divided in two to provide four experimental groups: non-stress and stressed rats with intraovarian administration of saline and

http://joe.endocrinology-journals.org
https://doi.org/10.1530/JOE-18-0357

non-stress and stressed rats with intraovarian treatment with RFRP-3. Stress exposure was designed according to Dorfman *et al.* (2003) and Bernuci *et al.* (2013); briefly, cold stress promotes ovarian morphological alterations related to a PCOS condition through activation of the ovarian sympathetic nerves (Bernuci *et al.* 2013). Minipump implantation was performed as previously reported (Lara *et al.* 2000). Animals were anesthetized with an i.m. dose of ketamine 60 mg/kg/xylazine 10 mg/kg solution under aseptic conditions. A transverse midlumbar incision, ~1.5 cm long, was made in the flank area on the one side of the animal to obtain access to the ovarian bursa. ALZET osmotic minipumps (model 2004, 0.25 µL/h, Alza Corp. Palo Alto, CA, USA) were connected to the underlying bursa of the right ovary with SILASTIC 0.64 mm ID × 1.19 mm OD CAT 508-003 (Dow Corning Corp, Midland, MI, USA) tubing for 28 days. Six of the control and six of the stressed rats were implanted with osmotic minipumps for intraovarian delivery of RFRP-3 at a concentration of 27 µg/µL. The remaining six controls and six stressed rats were implanted with an osmotic minipump filled with saline (0.9% NaCl). Rats subjected to stress were exposed to 4°C 5 days a week for 4 weeks. At the beginning of the 28-day treatment and to eliminate the possible contribution of the contralateral ovary to steroidogenesis, all control and peptide-treated animals were hemiovariectomized at the moment of the minipump implant as per Ricu *et al.* (2012).

At 28 days, the stress procedure and the effectiveness of the pump came to an end. Rats were maintained in their regular conditions for an additional 28 days (Bernuci *et al.* 2013), after which they were killed, and the ovary, hypothalamus (medio-basal hypothalamus-arcuate nucleus) and trunk blood were collected for analysis (Fig. 1). Ovaries were cut in half, one half was fixed with 10% paraformaldehyde solution for morphometric analysis and the other half was stored at −80°C for later mRNA extraction.

In vivo sympathetic stress

In order to study the contribution of the sympathetic system to the expression of RFRP-3 in the ovary in the first follicular cohort (a process that in the rat take 28 days) (Dorfman *et al.* 2003), immediately after the stress was finished, we collected the ovaries from an additional parallel group ($n = 5$ each) from control and stressed rats to determine any changes in RFRP protein expression via immunohistochemistry.

Real-time PCR

Total RNA was extracted as described by Chomczynski and Sacchi (1987) from the hypothalamus and half ovary from *in vivo* sympathetic stress group (control and stress). The primers used were from the published work of Geraghty *et al.* (2015) and a BLAST was performed to determine their specificity of the sequences. The sequences of the primers for real-time PCR are *Rfrp*: F: 5′-CCAAAGGTTTGGGAGAACAA-3′; R: 5′-GGGTCATGGCATAGAGCAAT-3′, mRNA *Rfrp* encodes the precursor of RFRP-3; *Npff1*: F: 5′-GGTCAGAA CGGGAGTGATGT-3′; R: 5′-AGGAAGATGAGCACGTA GGC-3′; *18S*: F: 5′-TCAAGAACGAAGTCGGAGG-3′; R: 5′-GGACATCTAAGGGCATCACA-3′. The PCR reaction mix contained 10 µL of Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies, Inc., California, USA); 0.25 µM of each 18S primer, 0.1 µM of each *Rfrp* primer, or 0.1 µM of each *Npff1* primer; 2 µL of cDNA and sterile water for a final volume of 20 µL. PCR reactions were performed using the IQ5 real-time thermocycler (BioRad) under the following conditions: 95°C for 20 s, 52°C for 20 s for *Rfrp* and *Npff1* (60°C for 18S), 72°C for 20 s and

Figure 1
Experimental design to study the effect of RFRP-3 on the stress-induced changes in ovary development.

http://joe.endocrinology-journals.org
https://doi.org/10.1530/JOE-18-0357

a final extension of 72°C for 10 min. All samples for RT-qPCR analysis were run in triplicate (with a no reverse transcriptase control as a negative control) and a mean value was used for the determination of mRNA levels. Relative quantification of *Rfrp* and *Npff1* mRNA levels was performed using the comparative Cq method with *18S* as the reference gene and with the formula $2^{-\Delta\Delta Cq}$.

Morphometric analysis

Ovaries previously fixed in 10% paraformaldehyde solution were embedded in paraffin, cut into 6 µm sections, and stained with hematoxylin and eosin. Morphometric analysis of whole ovaries was performed as previously described (Lara *et al.* 2000). All follicular structures were followed through all the slices. Briefly, preantral follicles correspond to follicles without any antral cavity, including primary and secondary follicles. Antral follicles were those with more than three healthy granulosa cell layers, the antrum and with a clearly visible nucleus of the oocyte. Cystic follicles were devoid of oocytes and displayed a large antral cavity, a well-defined thecal cell layer and a thin (mostly monolayer) granulosa cell compartment containing apparently healthy cells. Corpora lutea (CL) were characterized by their larger size, absence of oocyte and the presence of luteal cells instead of granulosa cells.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on 6 µm slices from fixed and paraffin-embedded ovaries, as previously described (Greiner *et al.* 2008) with rabbit anti-white-crowned sparrow GnIH, which recognizes the RFRP precursor polypeptide RFRP (Pac123/124, George Bentley) diluted 1:1000. This antiserum has been used to detect RFRP in rats in the past (Kriegsfeld *et al.* 2006, Kirby *et al.* 2009) in addition to hamsters, birds, monkeys and humans (Bentley *et al.* 2003, Ubuka *et al.* 2009*a*,*b*, McGuire & Bentley 2010).

Biotinylated goat anti-rabbit IgG (cat. no. sc-2040; Santa Cruz Biotechnology) was used as secondary antibody for RFRP. After incubating the samples with HRP-streptavidin, the ImmPACT VIP Peroxidase Substrate kit (Vector Laboratories, Inc.) was used as a chromogen. We performed two different negative controls for IHC, one was omission of the secondary antibody, and the second was preadsorption of the primary RFRP antiserum diluted 1:1000 with a solution of 1 µg/mL of peptide (NPFV another name for RFRP, Phoenix Pharmaceuticals Inc., USA) overnight at 4°C. Images were obtained using an Olympus light microscope (Olympus CX31, Tokyo, Japan) with Micrometrics SE Premium 4 software (ACCU-SCOPE, Inc., Commack, NY, USA). The results were analyzed by measuring the pixel intensity using the integrated optical density (IOD) function of the Image Pro Plus 6.0 program (Media Cybernetics, Inc., USA). Areas with strong immunoreactivity were compared with background staining adjacent to the area of interest in the same section. Similar sections of at least two slides per ovary and five ovaries per group were analyzed; the relative contribution of RFRP immunoreactivity to the total IOD (100%) of the whole ovary slice in the image was calculated for each follicle, separated out into data for preantral follicles, antral follicles and CL. The results were expressed as the mean ± the standard error of the mean (S.E.M.).

Plasma levels of steroid hormones

Plasma concentrations of steroid hormones testosterone, estradiol (E_2) and progesterone (P_4) were determined via EIA, following the manufacturer's instructions. The catalog numbers of the test kits were 11-TESHU-E01, 11-ESTHU-E01 and 11-PROHU-E01 for testosterone, E_2 and P_4, respectively (Alpco Diagnostic). Intra- and interassay variations were less than 5% for E_2, less than 6% for testosterone and less than 5% for P_4. The minimal detectable values were 10, 0.02 and 0.1 ng/mL, respectively.

Statistical analysis

Differences between the control and treatment groups were analyzed with paired *t*-test for the *in vitro* experiments and unpaired *t*-test for *in vivo* experiments. When four groups were analyzed, we used one-way ANOVA with Tukey's multiple comparisons test. Significance was set at $P<0.05$. The minimum number of rats to be used for the experiments was calculated as described in Zar (1984). Shapiro–Wilk test was applied to verify the normal distribution of data. All statistical analyses were performed using GraphPad Prism v5.0 software (GraphPad Software).

Results

Effect of RFRP-3 on progesterone and testosterone release from the ovary *in vitro*

RFRP-3 did not affect basal progesterone secretion from the ovary but significantly attenuated hCG-induced progesterone secretion (Fig. 2A). A similar effect of

http://joe.endocrinology-journals.org
https://doi.org/10.1530/JOE-18-0357

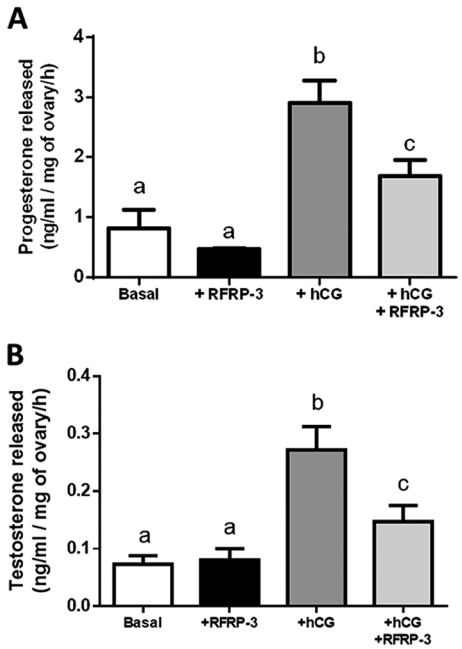

Figure 2
Progesterone (A) and testosterone (B) released to the incubation medium in basal conditions or in the presence of 10 ng/mL of RFRP-3, 2.5 IU of hCG or the combination of both. The ovaries of each rats were divided and incubated for 3 h to measure steroid secretion ($n=6$); mean ± S.E.M. Ordinary one-way ANOVA with Tukey's multiple comparisons test. Different letters represent significant differences ($P<0.05$) A: $F=2.925$, DFn = 3, DFd = 15; B: $F=0.9746$, DFn = 3, DFd = 16.

RFRP-3 was found regarding testosterone secretion. The peptide blocked the hCG-induced increase in testosterone secretion.

Stress-induced changes in hypothalamic and ovarian mRNA expression of RFRP and NPFF1

In order to determine the effect of stress on *Rfrp* and its receptor, we analyzed their expression in the hypothalamus and ovary. Figure 3A shows that the mRNAs for *Rfrp* and its receptor decreased in the mediobasal hypothalamus in response to stress. In the ovary, we found a decrease in the *Rfrp* mRNA and that of its receptor (Fig. 3B).

Localization of RFRP-3 in the ovary and the effect of 28-day stress in the expression in the rat ovary

The distribution of RFRP immunoreactivity in the ovary is shown in Fig. 4A. RFRP immunoreactivity is high in the interstitial tissue surrounding the antral follicles (Fig. 4A and C) and CL (Fig. 4B and D). It is also localized in granulosa cells of healthy antral follicles (Fig. 4C) and in the CL in low quantity (Fig. 4D). RFRP immunoreactivity

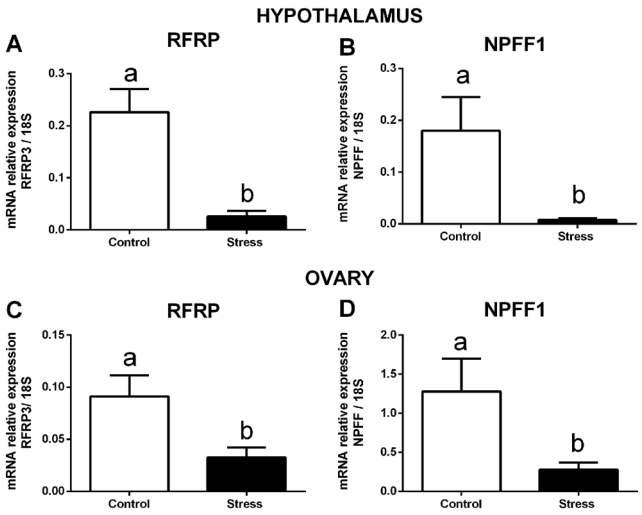

Figure 3
Changes in the mRNA expression of *Rfrp* and *Npff1* in hypothalamus (A and B) and the ovary (C and D). mRNA was normalized using ribosomal *18S* mRNA. Data correspond to the mean ± S.E.M. (A, B and D $n=5$; C $n=8$). Unpaired *t*-test. Different letters represent significant differences ($P<0.05$) A: $t=4.329$, $df=8$; B: $t=2.655$, $df=8$; C: $t=2.604$, $df=14$; D: $t=2.325$, $df=8$.

in the ovary of stressed rats was extremely low in all areas (Fig. 4E, F, G and H).

We performed semiquantitative IHC analysis for the peptide to determine the expression pattern of this peptide in the ovary. Figure 5 shows almost 90% decrease of the expression of the peptide after the exposure to sympathetic stress.

Effect of intraovarian *in vivo* administration of RFRP-3 on plasma steroid concentrations

Figure 6 shows the effect of *in vivo* RFRP-3 treatment on plasma steroid concentrations at the end of the experimental protocol. Cold stress caused an increase in plasma progesterone and the RFRP-3 administration blocked this effect (Fig. 6A). RFRP-3 treatment caused a decrease in testosterone in control rats but was not able to reverse the stress-induced increase in testosterone (Fig. 6B). No changes in plasma estradiol were detected in either control or cold stress-exposed rats in response to administration of RFRP3 (Fig. 6C).

Effect of sympathetic stress and RFRP-3 application to the ovary on estrus cycling activity

As shown in Fig. 7, the administration of RFRP-3 to control rats (stress or non- stress) induced a decrease in estrus cycling activity. Neither stress *per se* nor stress in

http://joe.endocrinology-journals.org
https://doi.org/10.1530/JOE-18-0357

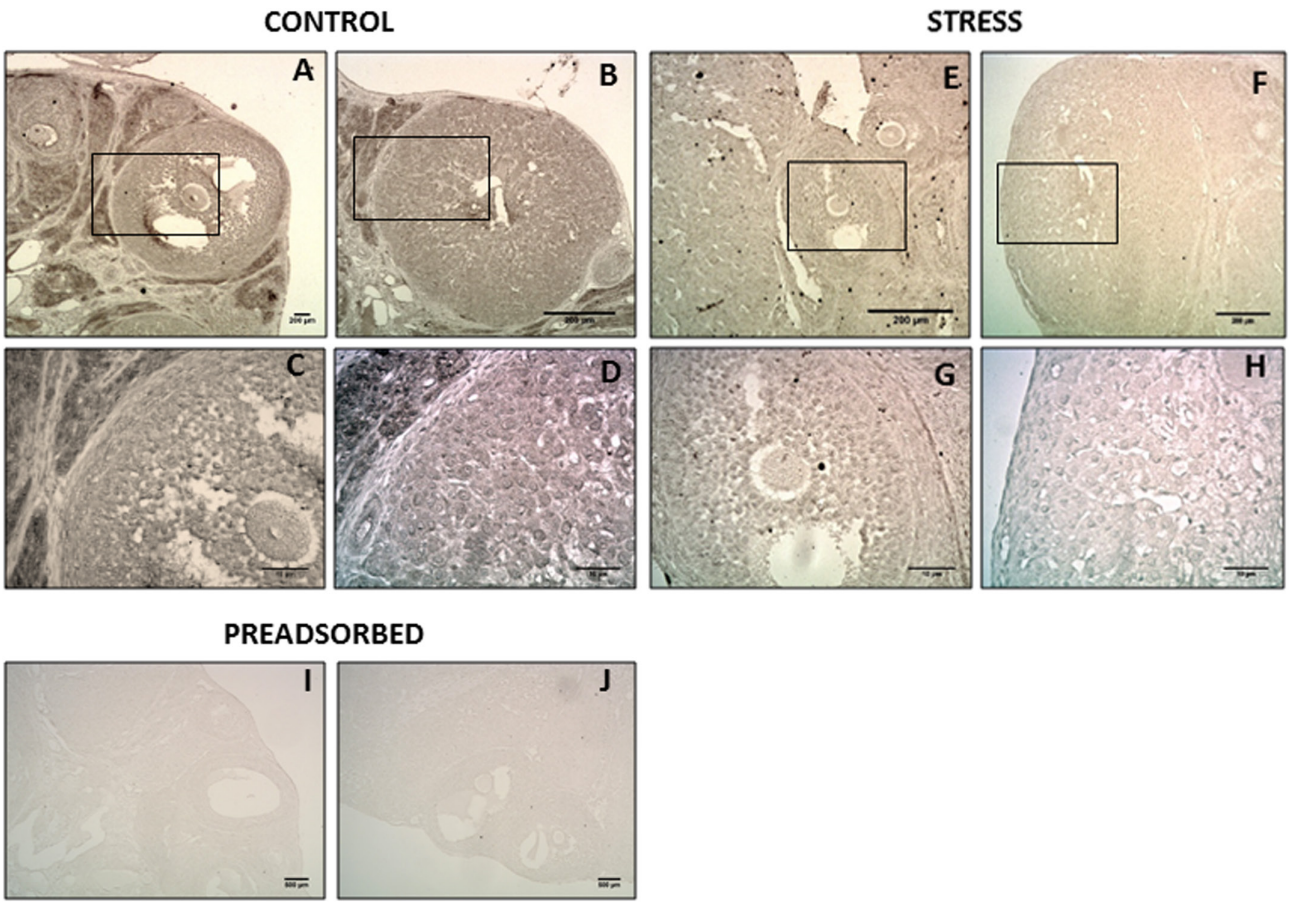

Figure 4
Distribution pattern of RFRP in the rat ovary. Immunohistochemical detection of RFRP in control ovary (A, B, C and D) and in the ovary of stressed rats for 28 days (E, F, G and H). Preadsorbed figures correspond to control (I) and stress (J) samples incubated with preadsorbed antibody as a negative control.

combination with RFRP-3 produced a decrease in estrus cycling activity.

Follicular dynamics in response to RFRP-3 *in vivo*

Morphometric analysis of the ovary is shown in Fig. 8. Non-stress rats exposed to intraovarian administration of RFRP-3 had similar numbers of secondary follicles relative to non-stress controls. Stressed rats had fewer secondary follicles, and RFRP-3 administration reversed this response (Fig. 8A). In terms of healthy antral follicles, a similar profile of follicular development appeared (Fig. 8B). Stress decreased the number of antral follicles, but this difference was partially reversed by the administration of RFRP-3. No changes were found in the atretic antral follicles population in response to RFRP-3 treatment or in response to stress (Fig. 8C). We also analyzed the cystic follicle population (Fig. 8D). In this case, administration of the peptide to stressed rats did not modify the number of cystic follicles, but the increase in the number of cysts following stress exposure was partially modified by RFRP3. Small changes in the CL population following intraovarian exposure to RFRP-3 to non-stressed animals or stressed animals were found. When results were studied by distribution of size (Fig. 8E), control, control plus RFRP3 and stressed rats presented a normal decline from smaller size to bigger size (these last represents the new CL), but there was an increase in the population of big CL and hence new CL because of the administration of RFRP3.

Discussion

Our data indicate that RFRP-3 can modify steroid secretion from the rat ovary *in vitro* and that it can participate in the regulation of a sympathetic stress-induced polycystic ovary phenotype. This stress paradigm

http://joe.endocrinology-journals.org
https://doi.org/10.1530/JOE-18-0357

caused a decrease in hypothalamic *Rfrp* mRNA expression and a decrease in *Npff1* mRNA expression in the ovary. In addition, RFRP immunoreactivity in the ovary was decreased 28 days into the stress paradigm. Finally, RFRP-3 application to the ovary *in vivo* and *in vitro* influenced ovarian steroidogenesis. We have previously used the *in vivo* protocol to administer growth factors, antisense oligonucleotides, peptides such as kisspeptin or pharmacological blockers like huperzine (Lara *et al.* 2000, Ricu *et al.* 2008, Fernandois *et al.* 2016, 2017, Urra *et al.* 2016); thus, we are confident of the effectiveness of this procedure. The present results strengthen the concept that RFRP-3 exerts local paracrine control of ovarian steroid synthesis and release and furthermore suggests that RFRP-3 may be involved in the development of PCOS. The model of chronic exposure to cold used here to induce PCOS-like physiology is powerful because of its ability to discriminate between a glucocorticoid-mediated stress condition and a NE-induced stress condition (Bhatnagar *et al.* 1995). In glucocorticoid-mediated stress conditions, the ovary responds by inhibiting steroid biosynthesis of progesterone, testosterone and estradiol secretion (Whirledge & Cidlowski 2017). In contrast, the stimulation of sympathetic nerves causes secretion of NE that acts on β_2 adrenergic receptors located at theca and granulosa cells. In this NE-induced stress condition, the ovary responds by increasing progesterone and testosterone secretion, without any effect on estradiol secretion (Lara *et al.* 2002, Greiner *et al.* 2005).

Data from our *in vitro* experiments indicate that exogenous RFRP-3 does not influence basal production of progesterone and testosterone from rat ovary, but it inhibits gonadotropin-stimulated (in this case, hCG-stimulated) production of these two steroids. Exactly where RFRP-3 is acting in the ovarian steroidogenic pathway of rats is not yet known, but it is likely, based on data from humans and birds (Oishi *et al.* 2012, Lynn *et al.* 2015) that RFRP-3 can act via reduction of steroidogenic acute regulatory (StAR) protein expression. This does not preclude the possibility that RFRP-3 can also act on specific enzymes in the steroidogenic pathway. In fact, our *in vivo* data demonstrating that RFRP-3 inhibited the stress-induced rise in progesterone (but not basal progesterone,

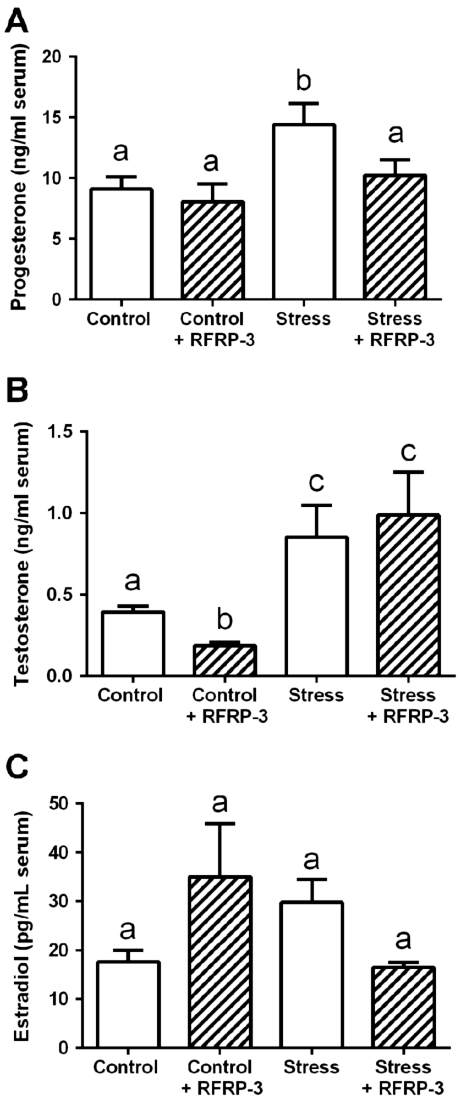

Figure 6
Plasma concentrations of progesterone (A), testosterone (B) and estradiol (C) after stress and the effect of RFRP-3 administration. Results correspond to six individual rats in each condition and are expressed as mean ± S.E.M. Ordinary one-way ANOVA with Tukey's multiple comparisons test. Different letters represent significant differences ($P<0.05$). A: $F=0.4340$, DFn = 3, DFd = 17; B: $F=8.965$, DFn = 3, DFd = 14.

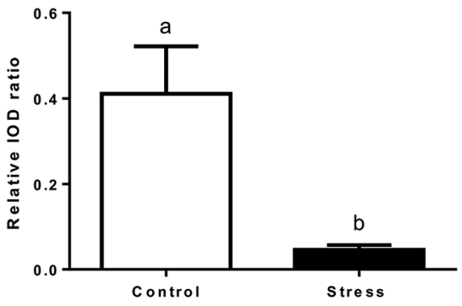

Figure 5
Immunoreactivity of RFRP in the ovary of control and 28-daystressed rats. The total integrated optical density (IOD) of the whole ovary slice in the image was considered 100% and then the contribution of each specific structure was calculated relative to the whole ovary. Similar sections of at least two slides per ovary and five ovaries per group were analyzed. Each group comprised five controls and five stressed female rats. The data are shown as the means ± S.E.M. Unpaired *t*-test. Different letters represent significant differences ($P<0.01$) $t=3.725$, $df=7$.

http://joe.endocrinology-journals.org
https://doi.org/10.1530/JOE-18-0357

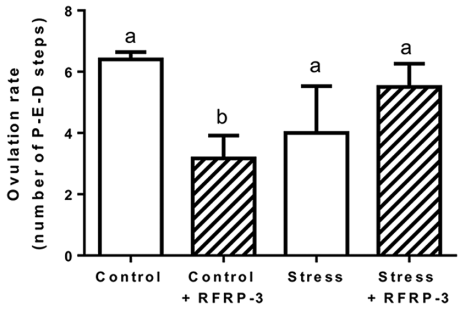

Figure 7
Number of estrus cycles. The number of estrus cycles was estimated as the regular passage from proestrus (P) to estrus (E) followed by diestrus (D) over the 56-day period. The groups correspond to Control (non-stress), 28-day Cold Stress exposure + 28 days resting (Stress) and 28-day Cold Stress exposure in the presence of 10 ng/mL of RFRP-3 locally delivered to the ovary by means of an osmotic minipump + 28-day resting. ($n = 6$); mean ± S.E.M. One-way ANOVA with Tukey's multiple comparisons test. Different letters represent significant differences ($P < 0.05$). $F = 1.022$, DFn = 3, DFd = 16.

Fig. 6A), and inhibited basal, but not the stress-induced increase in testosterone production, indicate that RFRP-3 might well be acting upon components of the steroidogenic pathway downstream from StAR in these circumstances. A second possibility is that sympathetic stress, through secretion of NE, is preferentially acting on testosterone biosynthesis as has been demonstrated previously (Cardinali *et al.* 1982). In support of this possibility, we found no differences in plasma estradiol concentrations.

Cold stress decreased the expression *Rfrp* and *Npff1* mRNA in hypothalamus and in the ovary. In addition, cold stress decreased RFRP immunoreactivity in the ovary. It is important to note that the strongest immunoreactivity for RFRP was in the interstitial theca cell layer – i.e., the cells involved in androgen section (Chang 2007, Bremer 2010), providing support for a primary effect of RFRP-3 as it also occurs with NE, in the regulation of plasma androgens. In support of this, treatment with RFRP-3 *in vitro* caused a decrease in androgen secretion. When these data are considered in light of the known actions of RFRP-3 (inhibition of gonadotropin secretion, reduction of ovarian steroidogenesis), the implication is that cold stress allows for an increase in gonadotropin release and ovarian steroidogenesis/androgen secretion via reduction in activity of hypothalamic and ovarian RFRP-3 or acting through a non-convergent downstream androgen biosynthetic pathway. Given that our cold stress paradigm induces a PCOS phenotype, and then based on our data, it is parsimonious to infer a role for RFRP-3 in the development of PCOS.

Notably, cold stress in the present study had the opposite effect on hypothalamic *Rfrp* from that seen in rats in response to restraint stress (Kirby *et al.* 2009,

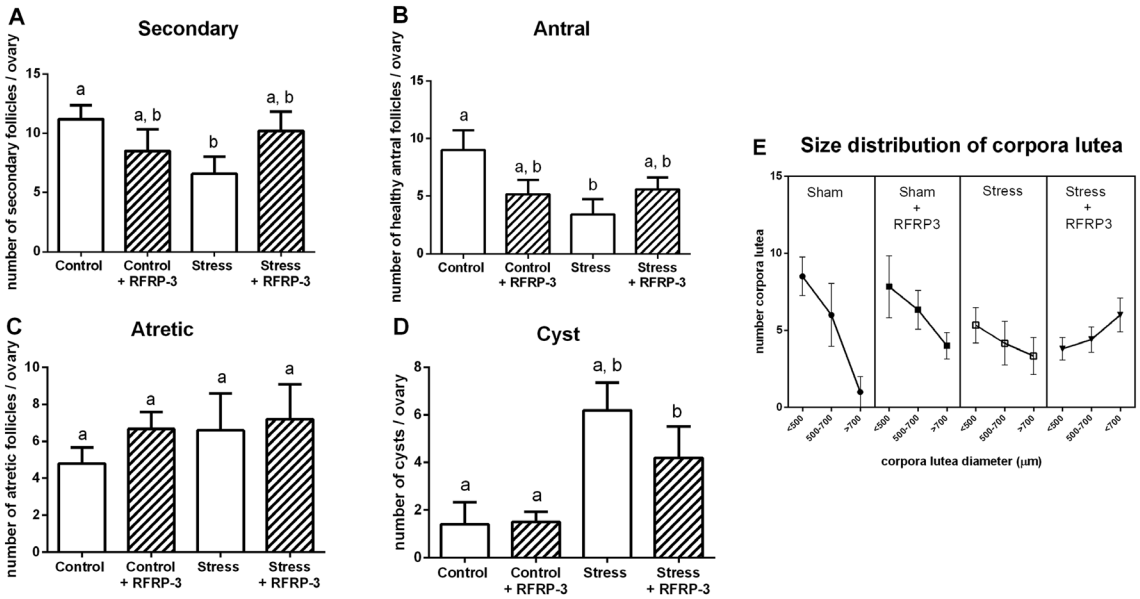

Figure 8
Ovarian follicular dynamic for the total follicular development studies. Number of secondary (A), antral (B), atretic (C), cyst (D) follicles and size distribution of corpora lutea (E). The groups correspond to Control (non-stress), 28-day Cold Stress exposure (Stress) and 28-day Cold Stress exposure in the presence of 10 ng/mL of RFRP-3 locally delivered to the ovary by an osmotic minipump. Rats were killed after 28 days of the stress and/or peptide administration was finished. ($n = 6$); mean ± S.E.M. One-way ANOVA with Tukey's multiple comparisons test. Different letters represent significant differences ($P < 0.05$).

http://joe.endocrinology-journals.org
https://doi.org/10.1530/JOE-18-0357

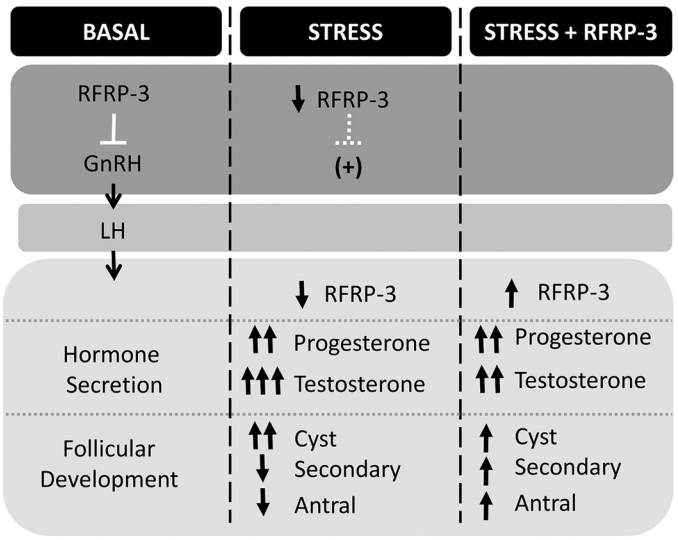

Figure 9
Model to explain the effect of RFRP-3 on the stress-induced polycystic phenotype in rat. The decrease in hypothalamic RFRP-3 by sympathetic stress results in decreased inhibition of GnRH secretion and thus an increase in LH secretion – leading to an LH-induced hyperandrogenism condition. Coincidentally, sympathetic stress that decreases ovarian RFRP-3 content. These changes act in concert to induce peripheral and local hyperandrogenism characteristically seen in the PCOS phenotype. Administration of RFRP-3 directly to the ovary can, at least partially, counteract these effects of sympathetic stress.

Geraghty *et al.* 2015). Different types of stressors have different effects on hypothalamic and gonadal *Rfrp* (increase or decrease) within an individual according to species, sex, reproductive status and ecological context of the stressor (Bentley *et al.* 2017). Our data support the idea that cold stress is interpreted physiologically in a way that is different from other stressors. Our findings highlight the need for specificity when using the term 'stress', but also highlight a complex and sometimes opposing role for RFRP in the brain and gonads in terms of regulation of steroidogenesis.

Administration of RFRP-3 decreased the number of estrus cycles that were observed over the 56 days of the experiment (28 days stress+28 days rest), and yet, there were more new CL present in stressed rats administered RFRP-3 after 28 days (Figs 7 and 8E). These findings indicate that ovarian RFRP-3 might simultaneously play a role in regulating estrus cycle frequency and in the recovery of ovulatory response. These effects are in addition to hypothalamic RFRP-3 influencing the timing of ovulation (Gibson *et al.* 2008). It is possible that the ovulatory response driven by RFRP-3 could be the result of an increased transition from secondary to antral follicles going to ovulation (both types of follicles were slightly decreased in number). The fact that RFRP-3 administration also partially rescued the effects of stress on development of secondary and antral follicles, and the stress effect on development of ovarian cysts, indicate a role for RFRP-3 in development of PCOS, but it is probable that this peptide is only part of a group of endocrine regulators of cyst formation, as per Dorfman *et al.* (2003) and Urra *et al.* (2016).

In sum, the present data strongly support a previously unidentified role for RFRP-3 in modulating ovarian physiology in response to sympathetic stress. This has allowed us to develop a hypothetical model (Fig. 9) for RFRP-3 in terms of how it might influence the development of a polycystic phenotype in rats. Clearly, further experiments are necessary to determine how RFRP-3 interacts with other known modulators of PCOS (Dorfman *et al.* 2003, Urra *et al.* 2016).

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This work was supported, in part, by a grant to support International Networking between International Research Centers REDES140061 (U de Chile, to H E L and UC Berkeley to G E B) and by Fondecyt grant 1170291 (to H E L). V S was also supported by a scholarship for Doctoral thesis support Conicyt N° 21110879.

Author contribution statement
V S performed most of the experimental work with rats and biochemical analysis. K W performed some qPCR and contributed to immunohistochemistry. R R performed the morphometric analysis. G B discussed the theoretical phases and experimental design of the work and contributed to draft the paper. H L conceived the study, directed the work and drafted the paper. All authors participated in the writing of the manuscript and approved of the final version.

References

Azziz R, Carmina E, Dewailly D, Diamanti-Kandarakis E, Escobar-Morreale HF, Futterweit W, Janssen OE, Legro RS, Norman RJ,

Taylor AE, *et al*. 2006 Position statement: Criteria for defining polycystic ovary syndrome as a predominantly hyperandrogenic syndrome: an Androgen Excess Society Guideline. *Journal of Clinical Endocrinology and Metabolism* **91** 4237–4245. (https://doi.org/10.1210/jc.2006-0178)

Barria A, Leyton V, Ojeda SR & Lara HE 1993 Ovarian steroidal response to gonadotropins and beta-adrenergic stimulation is enhanced in polycystic ovary syndrome: role of sympathetic innervation. *Endocrinology* **133** 2696–2703. (https://doi.org/10.1210/endo.133.6.8243293)

Benedict CR, Fillenz M & Stanford C 1979 Noradrenaline release in rats during prolonged cold-stress and repeated swim-stress. *British Journal of Pharmacology* **66** 521–524. (https://doi.org/10.1111/j.1476-5381.1979.tb13689.x)

Bentley GE, Perfito N, Ukena K, Tsutsui K & Wingfield JC 2003 Gonadotropin-inhibitory peptide in song sparrows (Melospiza melodia) in different reproductive conditions, and in house sparrows (Passer domesticus) relative to chicken-gonadotropin-releasing hormone. *Journal of Neuroendocrinology* **15** 794–802. (https://doi.org/10.1046/j.1365-2826.2003.01062.x)

Bentley GE, Ubuka T, McGuire NL, Calisi R, Perfito N, Kriegsfeld LJ, Wingfield JC & Tsutsui K 2009 Gonadotrophin-inhibitory hormone: a multifunctional neuropeptide. *Journal of Neuroendocrinology* **21** 276–281. (https://doi.org/10.1111/j.1365-2826.2009.01851.x)

Bentley GE, Wilsterman K, Ernst DK, Lynn SE, Dickens MJ, Calisi RM, Kriegsfeld LJ, Kaufer D, Geraghty AC, vivi DD, *et al*. 2017 Neural versus gonadal GnIH: are they independent systems? A mini-review. *Integrative and Comparative Biology* **57** 1194–1203. (https://doi.org/10.1093/icb/icx085)

Bernuci MP, Szawka RE, Helena CV, Leite CM, Lara HE & Anselmo-Franci JA 2008 Locus coeruleus mediates cold stress-induced polycystic ovary in rats. *Endocrinology* **149** 2907–2916. (https://doi.org/10.1210/en.2007-1254)

Bernuci MP, Leite CM, Barros P, Kalil B, Leoni GB, Del Bianco-Borges B, Franci CR, Szawka RE, Lara HE & Anselmo-Franci JA 2013 Transitory activation of the central and ovarian norepinephrine systems during cold stress-induced polycystic ovary in rats. *Journal of Neuroendocrinology* **25** 23–33. (https://doi.org/10.1111/j.1365-2826.2012.02373.x)

Bhatnagar S, Mitchell JB, Betito K, Boksa P & Meaney MJ 1995 Effects of chronic intermittent cold stress on pituitary adrenocortical and sympathetic adrenomedullary functioning. *Physiology and Behavior* **57** 633–639. (https://doi.org/10.1016/0031-9384(94)00161-8)

Bremer AA 2010 Polycystic ovary syndrome in the pediatric population. *Metabolic Syndrome and Related Disorders* **8** 375–394. (https://doi.org/10.1089/met.2010.0039)

Cardinali DP, Ritta MN & Gejman PV 1982 Norepinephrine stimulates testosterone aromatization and inhibits 5 alpha reduction via beta-adrenoceptors in rat pineal gland. *Molecular and Cellular Endocrinology* **28** 199–209. (https://doi.org/10.1016/0303-7207(82)90032-6)

Chang RJ 2007 The reproductive phenotype in polycystic ovary syndrome. *Nature Clinical Practice Endocrinology and Metabolism* **3** 688–695. (https://doi.org/10.1038/ncpendmet0637)

Chomczynski P & Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162** 156–159. (https://doi.org/10.1016/0003-2697(87)90021-2)

Dorfman M, Arancibia S, Fiedler JL & Lara HE 2003 Chronic intermittent cold stress activates ovarian sympathetic nerves and modifies ovarian follicular development in the rat. *Biology of Reproduction* **68** 2038–2043. (https://doi.org/10.1095/biolreprod.102.008318)

Ducret E, Anderson GM & Herbison AE 2009 RFamide-related peptide-3, a mammalian gonadotropin-inhibitory hormone ortholog, regulates gonadotropin-releasing hormone neuron firing in the mouse. *Endocrinology* **150** 2799–2804. (https://doi.org/10.1210/en.2008-1623)

Fernandois D, Lara HE & Paredes AH 2012 Blocking of beta-adrenergic receptors during the subfertile period inhibits spontaneous ovarian cyst formation in rats. *Hormone and Metabolic Research* **44** 682–687. (https://doi.org/10.1055/s-0032-1304607)

Fernandois D, Na E, Cuevas F, Cruz G, Lara HE & Paredes AH 2016 Kisspeptin is involved in ovarian follicular development during aging in rats. *Journal of Endocrinology* **228** 161–170. (https://doi.org/10.1530/JOE-15-0429)

Fernandois D, Cruz G, Na EK, Lara HE & Paredes AH 2017 Kisspeptin level in the aging ovary is regulated by the sympathetic nervous system. *Journal of Endocrinology* **232** 97–105. (https://doi.org/10.1530/JOE-16-0181)

Geraghty AC, Muroy SE, Zhao S, Bentley GE, Kriegsfeld LJ & Kaufer D 2015 Knockdown of hypothalamic RFRP3 prevents chronic stress-induced infertility and embryo resorption. *eLife* **4** e04316. (https://doi.org/10.7554/eLife.04316.001)

Gibson EM, Humber SA, Jain S, Williams WP 3rd, Zhao S, Bentley GE, Tsutsui K & Kriegsfeld LJ 2008 Alterations in RFamide-related peptide expression are coordinated with the preovulatory luteinizing hormone surge. *Endocrinology* **149** 4958–4969. (https://doi.org/10.1210/en.2008-0316)

Goldstein DS & Kopin IJ 2008 Adrenomedullary, adrenocortical, and sympathoneural responses to stressors: a meta-analysis. *Endocrine Regulations* **42** 111–119.

Goodarzi MO, Dumesic DA, Chazenbalk G & Azziz R 2011 Polycystic ovary syndrome: etiology, pathogenesis and diagnosis. *Nature Reviews Endocrinology* **7** 219–231. (https://doi.org/10.1038/nrendo.2010.217)

Greiner M, Paredes A, Araya V & Lara HE 2005 Role of stress and sympathetic innervation in the development of polycystic ovary syndrome. *Endocrine* **28** 319–324. (https://doi.org/10.1385/ENDO:28:3:319)

Greiner M, Paredes A, Rey-Ares V, Saller S, Mayerhofer A & Lara HE 2008 Catecholamine uptake, storage, and regulated release by ovarian granulosa cells. *Endocrinology* **149** 4988–4996. (https://doi.org/10.1210/en.2007-1661)

Heider U, Pedal I & Spanel-Borowski K 2001 Increase in nerve fibers and loss of mast cells in polycystic and postmenopausal ovaries. *Fertility and Sterility* **75** 1141–1147. (https://doi.org/10.1016/S0015-0282(01)01805-2)

Kirby ED, Geraghty AC, Ubuka T, Bentley GE & Kaufer D 2009 Stress increases putative gonadotropin inhibitory hormone and decreases luteinizing hormone in male rats. *PNAS* **106** 11324–11329. (https://doi.org/10.1073/pnas.0901176106)

Kriegsfeld LJ, Mei DF, Bentley GE, Ubuka T, Mason AO, Inoue K, Ukena K, Tsutsui K & Silver R 2006 Identification and characterization of a gonadotropin-inhibitory system in the brains of mammals. *PNAS* **103** 2410–2415. (https://doi.org/10.1073/pnas.0511003103)

Lara HE, Ferruz JL, Luza S, Bustamante DA, Borges Y & Ojeda SR 1993 Activation of ovarian sympathetic nerves in polycystic ovary syndrome. *Endocrinology* **133** 2690–2695. (https://doi.org/10.1210/endo.133.6.7902268)

Lara HE, Dissen GA, Leyton V, Paredes A, Fuenzalida H, Fiedler JL & Ojeda SR 2000 An increased intraovarian synthesis of nerve growth factor and its low affinity receptor is a principal component of steroid-induced polycystic ovary in the rat. *Endocrinology* **141** 1059–1072. (https://doi.org/10.1210/endo.141.3.7395)

Lara HE, Dorfman M, Venegas M, Luza SM, Luna SL, Mayerhofer A, Guimaraes MA, Rosa ESAA & Ramirez VD 2002 Changes in sympathetic nerve activity of the mammalian ovary during a normal estrous cycle and in polycystic ovary syndrome: studies on norepinephrine release. *Microscopy Research and Technique* **59** 495–502. (https://doi.org/10.1002/jemt.10229)

Lynn SE, Perfito N, Guardado D & Bentley GE 2015 Food, stress, and circulating testosterone: cue integration by the testes, not the brain, in male zebra finches (Taeniopygia guttata). *General and*

Comparative Endocrinology **215** 1–9. (https://doi.org/10.1016/j.ygcen.2015.03.010)

McGuire NL & Bentley GE 2010 Neuropeptides in the gonads: from evolution to pharmacology. *Frontiers in Pharmacology* **1** 114.

Oishi H, Klausen C, Bentley GE, Osugi T, Tsutsui K, Gilks CB, Yano T & Leung PC 2012 The human gonadotropin-inhibitory hormone ortholog RFamide-related peptide-3 suppresses gonadotropin-induced progesterone production in human granulosa cells. *Endocrinology* **153** 3435–3445. (https://doi.org/10.1210/en.2012-1066)

Ricu M, Paredes A, Greiner M, Ojeda SR & Lara HE 2008 Functional development of the ovarian noradrenergic innervation. *Endocrinology* **149** 50–56. (https://doi.org/10.1210/en.2007-1204)

Ricu MA, Ramirez VD, Paredes AH & Lara HE 2012 Evidence for a celiac ganglion-ovarian kisspeptin neural network in the rat: intraovarian anti-kisspeptin delays vaginal opening and alters estrous cyclicity. *Endocrinology* **153** 4966–4977. (https://doi.org/10.1210/en.2012-1279)

Rizwan MZ, Harbid AA, Inglis MA, Quennell JH & Anderson GM 2014 Evidence that hypothalamic RFamide related peptide-3 neurones are not leptin-responsive in mice and rats. *Journal of Neuroendocrinology* **26** 247–257. (https://doi.org/10.1111/jne.12140)

Singh P, Krishna A, Sridaran R & Tsutsui K 2011*a* Immunohistochemical localization of GnRH and RFamide-related peptide-3 in the ovaries of mice during the estrous cycle. *Journal of Molecular Histology* **42** 371–381. (https://doi.org/10.1007/s10735-011-9340-8)

Singh P, Krishna A & Tsutsui K 2011*b* Effects of gonadotropin-inhibitory hormone on folliculogenesis and steroidogenesis of cyclic mice. *Fertility and Sterility* **95** 1397–1404. (https://doi.org/10.1016/j.fertnstert.2010.03.052)

Sverrisdottir YB, Mogren T, Kataoka J, Janson PO & Stener-Victorin E 2008 Is polycystic ovary syndrome associated with high sympathetic nerve activity and size at birth? *American Journal of Physiology: Endocrinology and Metabolism* **294** E576–E581. (https://doi.org/10.1152/ajpendo.00725.2007)

Tsutsui K 2009 A new key neurohormone controlling reproduction, gonadotropin-inhibitory hormone (GnIH): biosynthesis, mode of action and functional significance. *Progress in Neurobiology* **88** 76–88. (https://doi.org/10.1016/j.pneurobio.2009.02.003)

Tsutsui K, Bentley GE, Ubuka T, Saigoh E, Yin H, Osugi T, Inoue K, Chowdhury VS, Ukena K, Ciccone N, *et al*. 2007 The general and comparative biology of gonadotropin-inhibitory hormone (GnIH). *General and Comparative Endocrinology* **153** 365–370. (https://doi.org/10.1016/j.ygcen.2006.10.005)

Ubuka T, Lai H, Kitani M, Suzuuchi A, Pham V, Cadigan PA, Wang A, Chowdhury VS, Tsutsui K & Bentley GE 2009*a* Gonadotropin-inhibitory hormone identification, cDNA cloning, and distribution in rhesus macaque brain. *Journal of Comparative Neurology* **517** 841–855. (https://doi.org/10.1002/cne.22191)

Ubuka T, Morgan K, Pawson AJ, Osugi T, Chowdhury VS, Minakata H, Tsutsui K, Millar RP & Bentley GE 2009*b* Identification of human GnIH homologs, RFRP-1 and RFRP-3, and the cognate receptor, GPR147 in the human hypothalamic pituitary axis. *PLoS ONE* **4** e8400. (https://doi.org/10.1371/journal.pone.0008400)

Urra J, Blohberger J, Tiszavari M, Mayerhofer A & Lara HE 2016 In vivo blockade of acetylcholinesterase increases intraovarian acetylcholine and enhances follicular development and fertility in the rat. *Scientific Reports* **6** 30129. (https://doi.org/10.1038/srep30129)

Whirledge S & Cidlowski JA 2017 Glucocorticoids and reproduction: traffic control on the road to reproduction. *Trends in Endocrinology and Metabolism* **28** 399–415. (https://doi.org/10.1016/j.tem.2017.02.005)

Zar JH 1984 *Biostatistical Analysis*. Englewood Cliffs, NJ, USA: Prentice Hall.

Received in final form 17 July 2018
Accepted 31 July 2018
Accepted Preprint published online 2 August 2018