Direct stimulatory effect of ghrelin on pituitary release of LH through a nitric oxide-dependent mechanism that is modulated by estrogen

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Abstract

Ghrelin, a gut peptide with key actions on food intake and GH secretion, has been recently recognized as potential regulator of reproductive function. Thus, in adult female rats, ghrelin has been proven to modulate GnRH/LH secretion, with predominant inhibitory effects *in vivo*. We analyze herein potential direct pituitary effects of ghrelin on basal and GnRH-stimulated gonadotropin secretion in prepubertal female rats, and its interplay with ovarian inputs, nitric oxide (NO), and hypothalamic differentiation. In the experimental setting, pituitaries from intact and ovariectomized prepubertal female rats were challenged with ghrelin *in vitro* and LH secretion was monitored. Our results demonstrate that 1) ghrelin consistently stimulated *in vitro* pituitary LH secretion under different experimental conditions; 2) the sensitivity to ghrelin, expressed either as the minimal effective dose or the amplitude of the LH response, was modulated by ovarian inputs; 3) the blockade of estrogen action significantly augmented the stimulatory effect of ghrelin; 4) the stimulatory effect of ghrelin on LH secretion required proper NO synthesis; and 5) the ability of ghrelin to elicit LH secretion *in vitro* was preserved after alteration (masculinization) of brain sexual differentiation. Overall, our present data reinforce the concept that ghrelin participates in the control of LH secretion, with potential stimulatory actions at the pituitary level that require the presence of NO and are modulated by ovarian signals.

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Introduction

Although it has been long known that conditions of negative energy balance are frequently linked to lack of puberty onset and reproductive failure, only recently the mechanisms involved in the coupling of reproductive function and body energy stores have been partially elucidated (for a review, see Fernández-Fernández *et al.* 2006). In this context, recent evidence has demonstrated that central and peripheral endocrine signals governing energy homeostasis, such as the adipocyte-derived hormone leptin, the gastrointestinal-secreted molecule polypeptide YY_{3–36}, the neuropeptide Y (NPY), and orexins, are also involved in the control of reproductive function by acting at different levels of hypothalamic–pituitary–gonadal axis (Kalra & Crowley 1992, Pu *et al.* 1998, Casanueva & Diéguez 1999, Tamura *et al.* 1999, Ahima *et al.* 2000, Tena-Sempere & Barreiro 2002, Fernández-Fernández *et al.* 2005a). Proper reproductive function requires the precise regulation of gonadotropin secretion, which is achieved via complex interactions

between gonadotrophin-releasing hormone (GnRH), other hypothalamic peptides (Evans 1999, Moore *et al.* 2003), local pituitary signals (such as activins, inhibins, and follistatin; Meunier *et al.* 1988, Kogawa *et al.* 1991), and gonadal-derived steroids and peptides. In addition, it is known that sexual differentiation of the neuronal circuitry controlling gonadotropin secretion in adult age occurs in rodents during the neonatal period and is steroid dependent (Barracough 1961, Gorski 1963, 1990). This differentiation can be impaired by exogenous administration of sexual steroids (Barracough 1961, Gorski 1963, 1990, Bellido *et al.* 1985, Pinilla *et al.* 1992).

Ghrelin, a 28-amino acid peptide with an essential n-octanoylation at serine 3, is mainly expressed in stomach (Kojima *et al.* 1999, 2001) and stimulates food intake and growth hormone (GH) secretion in humans and rats (Kojima *et al.* 1999, 2001, Seoane *et al.* 2000, Wren *et al.* 2000, Ahnfelt-Ronne *et al.* 2001, Arvat *et al.* 2001a, 2001b, Ghigo *et al.* 2001, Hataya *et al.* 2001, Tayaka *et al.* 2001, Pinilla *et al.* 2003). These biological

effects are conducted through its interaction with the GH secretagogue receptor (GHS-R), a member of the large family of G-protein coupled, seven transmembrane domain receptors. Two GHS-R subtypes, generated by alternative splicing of a single gene, have been described so far: the full-length type 1a receptor and the truncated GHS-R type 1b; the GHS-R1a being the functionally active, signal transducing form of the receptor (Howard *et al.* 1996, McKee *et al.* 1997). In addition to the important role of ghrelin in the control of GH secretion and energy homeostasis, ghrelin carries out a plethora of endocrine and non-endocrine biological actions (Korbonits *et al.* 2004, van der Lely *et al.* 2004).

In the context of the proven role of ghrelin as a potent orexigenic hormone involved in the long-term control of body weight (Wren *et al.* 2000, Korbonits *et al.* 2004), recent data have suggested role of ghrelin in the regulation of reproduction, with a predominantly inhibitory effect upon reproductive function in primate and rodent species. Thus, expression of ghrelin has been demonstrated in human and rodent placenta, and ghrelin has been reported to inhibit early embryo development *in vitro* (Gualillo *et al.* 2001, Kawamura *et al.* 2003), and pregnancy outcome *in vivo* (Fernández-Fernández *et al.* 2005b). In addition, acute and chronic administration of ghrelin was shown to suppress luteinizing hormone (LH) secretion *in vivo* in prepubertal and adult male and female rats and monkeys (Furuta *et al.* 2001, Fernández-Fernández *et al.* 2004, 2005b, 2005c, Vulliémoz *et al.* 2004, Martini *et al.* 2006) and to decrease *in vitro* GnRH secretion (Fernández-Fernández *et al.* 2005c) and LH responsiveness to GnRH (Fernández-Fernández *et al.* 2004, 2005c). Moreover, ghrelin was able to inhibit testosterone secretion *in vivo* and *in vitro* (Tena-Sempere *et al.* 2002, Fernández-Fernández *et al.* 2005b) and partially prevented the normal timing of balanopreputial separation, an external index of puberty onset, in rats (Fernández-Fernández *et al.* 2005b, Martini *et al.* 2006). Finally, expression of ghrelin and its cognate receptor has been demonstrated in rat and human gonads (Barreiro *et al.* 2002, 2003, Tena-Sempere *et al.* 2002, Gaytán *et al.* 2003, 2004) and GHS-R1a mRNA has been detected in pituitaries from adult female rats in all phases of estrous cycle (Fernández-Fernández *et al.* 2005c). Finally, in conditions of negative energy balance, such as fasting or anorexia nervosa, high plasma levels of ghrelin are accompanied by decreased LH secretion (Camina *et al.* 2003, Misra *et al.* 2005), which is compatible with its potential inhibitory effect upon reproductive function.

Despite its suggested inhibitory role in the central control of the gonadotropic axis (Furuta *et al.* 2001, Fernández-Fernández *et al.* 2004, 2005c, Vulliémoz *et al.* 2004), the potential contribution of direct pituitary effects of ghrelin in the control of gonadotropin secretion remains scarcely studied, and data so far available

evidenced either no effects (Korbonits *et al.* 2004, van der Lely *et al.* 2004) or paradoxical stimulatory actions on basal LH and FSH secretion in prepubertal male rats and in adult cyclic female rats (Fernández-Fernández *et al.* 2004, 2005c). In this scenario, the aim of present work was to provide further information of the role of ghrelin in the direct control of LH secretion at pituitary level in prepubertal rats, with special attention to the possible role of ovarian inputs, nitric oxide (NO), and brain sexual differentiation in the control of ghrelin effects on pituitary LH release.

Material and Methods

Animals and drugs

Prepubertal (30 days) female Wistar rats were used. The rats were housed (four per cage) under controlled conditions of light (light on from 0500 to 1900 h) and temperature (22 °C), with free access to tap water and food available *ad libitum*. All the experiments were conducted under the approval of the Committee of Animal Experimentation of the University of Córdoba, and in accordance with the NIH Guide for Care and Use of Laboratory Animals.

Ghrelin was obtained from Bachem (Barcelona, Spain). The pure antiestrogen ICI 182 780 (7-[9-[4,4,5,5,5-pentafluoropentyl)sulfinyl] nonyl]-estra-1,3,5(10)-triene-3,17diol) was obtained from Tocris (Madrid, Spain). GnRH, 17 β -estradiol 3-benzoate (EB), and the inhibitor of NO synthase N_w-nitro-L-arginine methyl ester (L-NAME) were obtained from Sigma. ICI 182 780 was dissolved initially in a few drops of dimethylsulfoxide and thereafter was dissolved in saline up to the working concentration; the injection volume was 0.1 ml. EB was dissolved in olive oil; the injection volume was 0.1 ml. Ghrelin, GnRH, and L-NAME were dissolved in Dulbecco's Modified Eagle's Medium (DMEM; BioWhittaker; Verriers, Belgium) immediately before use.

Experiments

In order to detect a primary action of ghrelin in the regulation of basal LH secretion in prepubertal female rats, in Experiment 1, 23-day-old females were ovariectomized or sham-ovariectomized, and 7 days later were humanely killed by decapitation (between 1100 and 1200 h). The anterior pituitaries were obtained and placed in glass scintillation vials (one per vial) in a Dubnoff shaker at 37 °C under an atmosphere of 96% O₂–5% CO₂. Each vial contained 1 ml DMEM. After preincubation for 60 min, the medium was replaced by fresh medium alone or containing increasing doses of ghrelin (10^{−9}–10^{−6} M). Of note, such a range of doses were selected on the basis of previous references testing direct effects of ghrelin on anterior pituitary secretion (Kojima *et al.* 1999, Fernández-Fernández *et al.* 2004,

2005c). Medium samples were obtained at 60 and 120 min of the incubation period. Each group was composed of 8–12 pituitaries. On the basis of results from this experiment, an effective dose of ghrelin 10^{-6} M was set for the following experimental settings.

Results from Experiment 1 suggested that *in vitro* LH response to ghrelin was modulated by ovarian inputs. Since prepubertal ovaries secrete different steroids and peptides, we decided to analyze the possible selective role of estrogens. Thus, in Experiment 2, prepubertal female rats were subcutaneously injected between day 23 and 29 of age with ICI 182 780 (150 μ g/rat per day) or vehicle. The animals were humanely killed by decapitation 24 h after the last injection, and their pituitaries incubated, as described for Experiment 1, in the presence of DMEM alone or containing 10^{-6} M ghrelin. Medium samples were obtained at 60 and 120 min of the incubation period. Each group consisted of 10–12 pituitaries. In addition, to analyze the potential role of ovarian inputs other than estrogen, in Experiment 3, 23-day-old female rats were ovariectomized and subcutaneously injected with EB (10 μ g/rat per day) or vehicle on days 3, 5, and 7 post-ovariectomy. The animals were humanely killed by decapitation 24 h after the last injection and their pituitaries incubated, as described for Experiment 1, in the presence of DMEM alone or 10^{-6} M ghrelin. Medium samples were obtained at 60 and 120 min of the incubation period. Each group was composed of ten pituitaries.

In order to detect a primary action of ghrelin on GnRH-stimulated LH secretion in prepubertal female rats, in Experiment 4, 23-day-old females were ovariectomized or sham-ovariectomized, and 7 days later were humanely killed by decapitation and their pituitaries incubated, as described for Experiment 1, in the presence of DMEM alone, ghrelin (10^{-6} M), GnRH (10^{-7} M) or ghrelin (10^{-6} M) plus GnRH (10^{-7} M). Medium samples were obtained at 60 and 120 min of the incubation period. Each group consisted of 8–12 pituitaries.

Since some of the pituitary effects of ghrelin have been reported to require the presence of NO (Gaskin *et al.* 2003, Pinilla *et al.* 2003), we analyze the potential participation of NO in the direct stimulatory effect of ghrelin on LH secretion. Thus, in Experiment 5, 23-day-old female rats were ovariectomized, and 7 days later were humanely killed by decapitation and their pituitaries incubated, as described for Experiment 1, in the presence of DMEM alone or medium containing ghrelin (10^{-6} M), L-NAME (10^{-4} M) or ghrelin (10^{-6} M) plus L-NAME (10^{-4} M). Medium samples were obtained at 60 and 120 min of the incubation period. Each group consisted of ten pituitaries.

Finally, in order to detect the influence of brain sexual differentiation on the effects of ghrelin in the control of LH secretion, in Experiment 6, female rats were subjected to a standard protocol of neonatal estrogenization (100 μ g

EB/rat on day 1, s.c.), and on day 30 *post partum*, the animals were humanely killed by decapitation, and the hypothalamus and pituitaries were incubated to monitor the effects of ghrelin on GnRH and LH secretion respectively. The hypothalami were rapidly excised and dissected out by a horizontal cut of ~ 2 mm depth with the following limits: 1 mm anterior from the optic chiasm, the posterior border of the mamillary bodies, and the hypothalamic fissures. Tissue samples were subsequently incubated in 250 μ l DMEM, in a Dubnoff shaker incubator under an atmosphere of 95% O₂ and 5% CO₂ at 37.5 °C. After a 30-min preincubation, the media were removed and the hypothalami were challenged for 30 min with ghrelin (10^{-6} M) or medium alone. At the end of incubation period, medium samples were boiled for 30 min to inactivate endogenous protease activity and stored at -80 °C until used for hormone determination. In addition, pituitaries were incubated, as described for Experiment 1, in the presence of DMEM alone or 10^{-6} M ghrelin. Medium samples were obtained at 60 and 120 min of the incubation period. Each group contained 10–12 hypothalami or pituitaries.

LH and GnRH determinations

LH levels were measured in 5–50 μ l samples by a double-antibody method using a RIA kit supplied by NIDDK (Bethesda, MD, USA). Rat LH-I-10 was labeled with ¹²⁵I using the iodogen method, following the instructions of the manufacturer (Pierce, Rockford, IL, USA) and hormone concentrations were expressed using the RP LH-RP3 as standard. Intra- and interassay variations were 8 and 10% respectively. The sensitivity of the assay was 5 pg/tube. In addition, GnRH concentrations in the incubation media from hypothalamic explants were measured in 100 μ l aliquots using a commercial RIA kit purchased from Peninsula Laboratories Inc (Bachem), following the instructions of the manufacturer. The sensitivity of the assay was 1 pg/tube and the intra-assay variation was <10%. Samples from each experiment were measured in the same assay.

Statistical analysis

Values are expressed as means $\pm$ S.E.M. Results were analyzed for statistically significant differences by means of ANOVA followed by Student–Newman–Keuls multiple range test (SigmaStat 2.0, Jandel Corp., San Rafael, CA, USA). In detail, one-way or two-way repeated measures (RM) ANOVA was applied for statistical comparison, as our studies involved subsequent LH determinations at 60 and 120 min after incubation in the presence of the testing compounds. Specifically, two-way RM ANOVA was used to evaluate the effect of ghrelin *in vitro* in the presence of additional

in vivo covariates, such as gonadectomy or steroid treatment. $P \leq 0.05$ was considered significant.

Results

Role of ovarian inputs in the effects of ghrelin on basal pituitary LH secretion

The dose-dependent effects of ghrelin on LH secretion directly at the pituitary level were first evaluated. In pituitary samples from intact females, ghrelin significantly stimulated LH secretion only at the dose of 10^{-6} M, both at 60 and 120 min of incubation (Fig. 1A). In contrast, an increased sensitivity in LH responses to ghrelin became apparent after ovariectomy, as significant increases in LH secretion were observed after challenge with 10^{-8} M (at 120 min) and 10^{-7} M (at 60 and 120 min) ghrelin of pituitaries from gonadectomized prepubertal rats (Fig. 1B). Due to the accumulative nature of our incubation system, LH levels at 120 min were significantly higher than those at 60 min in all the experimental groups.

Figure 1 LH concentrations in the media 60 and 120 min after incubation of pituitaries from 30-day-old intact and ovariectomized female rats. Incubations were carried out in the presence of ghrelin (10^{-9} – 10^{-6} M) or DMEM alone. Values are expressed as means $\pm$ S.E.M. ($n=8$ –12/group). ** $P \leq 0.01$ versus DMEM at the corresponding time-point; ^a $P \leq 0.01$ versus corresponding values at 60 min (one-way RM ANOVA followed by Student–Newman–Keuls multiple range test).

To further explore the involvement of ovarian signals in the modulation of pituitary responsiveness to ghrelin, *in vitro* tests were conducted using pituitaries from rats treated with the antiestrogen ICI 182 780 or gonadectomized and supplemented with estradiol. Treatment with ICI 182 780 was effective to block endogenous estrogen action, since serum LH concentrations were significantly increased at the end of treatment regimen (2.46 ± 0.44 vs 0.13 ± 0.01 ng/ml; $P \leq 0.01$). In keeping with results from Experiment 1, 10^{-6} M ghrelin significantly stimulated LH release *in vitro*, at 60 and 120 min, by pituitaries from vehicle- and ICI 182 780-treated animals (Fig. 2A). However, the magnitude of LH responses to ghrelin was significantly augmented in the ICI-treated group, at 60 and 120 min of incubation period (Fig. 2A).

In turn, analysis of LH responses to ghrelin in pituitaries from ovariectomized rats, replaced or not with estradiol, revealed that ghrelin elicited LH release by pituitaries from ovariectomized and ovariectomized+estradiol-treated female rats (Fig. 2B). The effectiveness of estradiol treatment in this setting was confirmed by the decrease in serum LH concentrations in terminal trunk blood samples (1.49 ± 0.24 vs 16.83 ± 1.26 ng/ml in ovariectomized rats; $P \leq 0.01$). Of note, pituitaries from ovariectomized(estradiol-treated rats released significantly more LH than pituitaries from ovariectomized animals treated with vehicle (Fig. 2B). Given these 'basal' differences, in addition to net (absolute) responses, the effects of ghrelin on LH secretion were also expressed as percentage of increase over corresponding control values from pituitaries incubated with medium alone. As shown in Fig. 3, relative LH responses to ghrelin appeared increased after ovariectomy, slightly decreased after estradiol replacement, and clearly enhanced after blockade of endogenous estrogen by ICI 182 780.

Effect of ghrelin on GnRH-stimulated LH secretion

The interplay between ghrelin and GnRH secretion was explored in prepubertal female rats. GnRH-stimulated LH release by pituitaries from intact and ovariectomized females, at 60 and 120 min of the incubation (Fig. 4). The stimulatory effect of GnRH was potentiated by ghrelin in pituitaries obtained from intact and ovariectomized females (Fig. 4), a phenomenon that was significant at 120 min in the intact group, and at both 60 and 120 min of incubation in ovariectomized rats (Fig. 4).

Role of NO in the stimulatory effect of ghrelin on LH secretion

Blockade of endogenous NO synthases by L-NAME had no effect on LH secretion by pituitaries from ovariectomized rats. However, the stimulatory effect of ghrelin

Figure 2 A (upper panel) LH concentrations in the media 60 and 120 min after incubation of pituitaries from 30-day-old intact female rats treated between days 23 and 30 with vehicle (–) or ICI 182 780 (+) (150 µg/rat per day). B (lower panel) LH concentrations in the media 60 and 120 min after incubation of pituitaries from 31-day-old ovariectomized female rats. Ovariectomy was carried out on day 23 and ovariectomized rats were treated on days 26, 28, and 30 with vehicle (–) or estradiol benzoate (+) (10 µg/rat per day). Incubations were carried out in the presence of ghrelin (10⁻⁶ M; solid bars) or DMEM alone (open bars). Values are expressed as means ± s.e.m. (n=10–12/group). **P≤0.01 versus DMEM, ^aP≤0.01 versus corresponding animals not treated with ICI 182 780 or estradiol benzoate *in vivo*; ^bP≤0.01 versus corresponding values at 60 min (two-way RM ANOVA followed by Student–Newman–Keuls multiple range test).

on LH secretion was blunted in the presence of L-NAME, at 60 and 120 min of the incubation period (Fig. 5).

Effects of neonatal estrogenization on ghrelin effects on GnRH and LH secretion

Female rats subjected to neonatally estrogenization showed a significant reduction in ovarian weights and serum LH levels on day 30 *post partum* (Table 1).

Figure 3 Effects of ghrelin (10⁻⁶ M) on LH release after 60 and 120 min of the incubation period from intact, ovariectomized (OVX), OVX plus estradiol benzoate (EB) or intact rats treated with ICI 182 780. Values are expressed as percentage of increase over LH concentrations measured in absence of ghrelin. Groups with different superscript letters are statistically different (ANOVA followed by Student–Newman–Keuls multiple range test).

Ghrelin, at the dose of 10⁻⁶ M, was unable to modify GnRH release by hypothalamic explants from control or neonatally estrogenized female rats *ex vivo* (Table 2). Concerning pituitary effects, *in vitro* basal pituitary LH secretion was significantly reduced in neonatally estrogenized female rats. Yet, the ability of ghrelin to induce significant stimulatory responses in terms of LH secretion was persistently observed in estrogenized female rats, at 60 and 120 min of incubation. Nonetheless, in this experiment, statistical analyses by two-way RM ANOVA, using *in vivo* treatment (with or without estradiol) and *in vitro* conditions (with or without ghrelin) as variables, evidenced that, at both 60 and 120 min of incubation, the response to ghrelin was attenuated in EB-treated female rats (Fig. 6).

Discussion

Our present results reinforce previously published data pointing out that ghrelin conducts specific regulatory effects upon the GnRH/LH axis (Barreiro & Tena-Sempere 2004); yet, they provide novel evidence for the multifaceted mode of action of ghrelin on the gonadotropic system. The most relevant findings of the present experiments can be summarized as follows: (1) ghrelin consistently stimulated pituitary LH secretion *in vitro* in a wide diversity of experimental conditions in prepubertal female rats; (2) the sensitivity to ghrelin, expressed as either the minimal effective dose or the amplitude of LH responses, was modulated by ovarian inputs; (3) the blockade of estrogen action significantly augmented the stimulatory effect of ghrelin; (4) the stimulatory effect of ghrelin on LH secretion required proper NO synthesis;

Figure 4 LH concentrations in the media 60 and 120 min after incubation of pituitaries from intact (left panel) and ovariectomized (right panel) females. Incubations were carried out in the presence of DMEM, ghrelin (10^{-6} M), GnRH (10^{-7} M), or ghrelin+GnRH. Values are expressed as means $\pm$ S.E.M. ($n=8-12$ /group). ** $P \leq 0.01$ versus corresponding DMEM groups, ^a $P \leq 0.01$ versus corresponding groups challenged with GnRH alone; ^b $P \leq 0.01$ versus corresponding values at 60 min (one-way RM ANOVA followed by Student–Newman–Keuls multiple range test).

and (5) ghrelin did not alter hypothalamic GnRH secretion *in vitro* in prepubertal female rat and its ability to stimulate pituitary LH secretion *in vitro* was preserved after neonatal estrogenization. The range of doses of ghrelin used in the present study was selected on the basis of previous studies testing its effects on anterior pituitary secretion (Kojima *et al.* 1999, Fernández-Fernández *et al.* 2004, 2005c). Of note, ghrelin input on the pituitary in physiologic conditions might theoretically derive from three different sources, i.e. systemic gut-derived ghrelin, hypothalamic-released peptide and locally produced ghrelin (Kojima *et al.* 1999, Caminos *et al.* 2003, Korbonits *et al.* 2004). This fact, together with potential tissue-specific changes in ghrelin expression depending on the stage of development, metabolic cues and pathologic conditions (van der Lely *et al.* 2004), make it difficult to estimate the actual ghrelin burden on the

Figure 5 LH concentrations in the media 60 and 120 min after incubation of pituitaries from 30-day-old ovariectomized rats in the presence of DMEM, ghrelin (10^{-6} M), L-NAME (10^{-4} M), or ghrelin + L-NAME. Ovariectomy was carried out on day 23. Values are expressed as means $\pm$ S.E.M. ($n=10$ /group). ** $P \leq 0.01$ versus DMEM at the corresponding time-point; ^a $P \leq 0.01$ versus corresponding values at 60 min (one-way RM ANOVA followed by Student–Newman–Keuls multiple range test).

pituitary, but it is likely that its local levels are (much) higher than those detected in circulation.

Ghrelin effects on GnRH/LH release appear to be age and sex dependent. Thus, detail comparison with previously reported data in adult cyclic rats (Fernández-Fernández *et al.* 2005c) identifies two clear differences: (i) ghrelin inhibits GnRH release in adult but not in prepubertal female rats and (ii) ghrelin blunts GnRH-stimulated LH-stimulated secretion in adult female rats, but apparently potentiates it in prepubertal females. Assumedly, elucidation of the mechanisms for these differences, and their possible role in pubertal development, need further studies. Nonetheless, it is to be stressed that previous evidence has strongly suggested the potential involvement of ghrelin in the regulation of the gonadotropic axis at puberty in the rat (Fernández-Fernández *et al.* 2005b, Martini *et al.* 2006).

In previous studies, we have demonstrated that ghrelin stimulates gonadotropin secretion by pituitaries from intact prepubertal male rats (Fernández-Fernández *et al.* 2004) and adult cyclic females (Fernández-Fernández *et al.* 2005c). Present data showed that ghrelin consistently stimulates pituitary LH secretion in different prepubertal female models such as intact animals,

Table 1 Luteinizing hormone (LH) concentrations and ovarian weights in 30-day-old female rats injected on day 1 with vehicle or estradiol benzoate (females: 100 μ g/rat; males: 500 μ g/rat).

Treatment	LH (ng/ml)	Ovary (mg)
Vehicle	0.69 ± 0.19 (21)	7.54 ± 0.20 (25)
EB	0.12 ± 0.01 (21)*	1.27 ± 0.09 (25)*

Values are expressed as means $\pm$ S.E.M. Number of animals per group are indicated in parentheses. * $P \leq 0.01$ (Student's test).

Table 2 Effects of ghrelin (10^{-6} M) on gonadotrophin-releasing hormone (GnRH) release (pg/hypothalamus/30 min) by hypothalamic explants obtained in 30-day-old females rats neonatally injected on day 1 of life with vehicle or estradiol benzoate (100 µg/rat).

	EMEM	Ghrelin
Control females	5.92 ± 0.62 (11)	6.97 ± 0.87 (12)
Estrogenized females	4.40 ± 0.57 (11)	6.80 ± 1.00 (12)

Values are expressed as means $\pm$ S.E.M. Number of determination per group are indicated in parentheses. * $P \leq 0.01$ (Student's test).

females treated with an antiestrogen, ovariectomized females submitted or not to estradiol replacement, and in intact females subjected to an effective protocol of neonatal estrogenization. These results evidence that the ability of ghrelin to elicit LH secretion directly at the pituitary level can manifest regardless of the prevailing pituitary LH content, the ovarian inputs, and the neonatal steroid milieu. It is worthy noting that ghrelin has been reported to increase LH secretion also by dispersed pituitary cells from the goldfish (Unniappan & Peter 2004), suggesting the conservation of this function during evolution.

Previous studies have repeatedly indicated that the effects of different factors involved in the control of LH secretion are dependent on the steroid milieu. For instance, the stimulation of LH secretion after activation of receptors for excitatory amino acids with *N*-methyl-D-aspartic acid (NMDA; as agonist of NMDA receptors) or AMPA (as agonist of non-NMDA receptors) requires the presence of estradiol (Brann & Mahesh 1995, Ping *et al.* 1997, González *et al.* 1999a). Our present results point out that the stimulatory effect of ghrelin on LH secretion at pituitary level is influenced by ovarian inputs. This

Figure 6 LH concentrations in the media 60 and 120 min after incubation of pituitaries with DMEM (open bars) or ghrelin (10^{-6} M; hatched bars). Female rats were injected on day 1 with vehicle (–) or estradiol benzoate (+). Values are expressed as means $\pm$ S.E.M. ($n = 10$ – 12 /group). ** $P \leq 0.01$ versus DMEM alone, ^a $P \leq 0.01$ versus non-estrogenized animals; ^b $P \leq 0.01$ versus corresponding values at 60 min (two-way RM ANOVA followed by Student–Newman–Keuls multiple range test).

contention is suggested by the increase in the sensitivity to ghrelin after ovariectomy and the blockade of endogenous estrogen by ICI treatment, as well as by the decrease in the responsiveness to ghrelin observed in ovariectomized-estradiol-treated females. The precise mechanism(s) whereby estrogen modulates pituitary responsiveness to ghrelin remain to be elucidated as, in principle, such an effect might derive from direct pituitary actions of estrogen and/or indirect effects mediated by changes in the prevailing GnRH input *in vivo*. In addition, the potential contribution of changes in the pituitary content of LH cannot be ruled out. Nonetheless, our current observations on the influence of estrogen on LH responses are in good agreement with the changes in pituitary responsiveness to ghrelin along the estrous cycle, as reported recently by our group (Fernández-Fernández *et al.* 2005c).

Present results are somewhat opposite to those previously reported by our group on the effects of ovarian inputs upon ghrelin effects on LH secretion (Fernández-Fernández *et al.* 2005c). Thus, in adult females, ovariectomy abolished the direct stimulatory effect of ghrelin on basal LH secretion; estradiol replacement being unable to rescue ghrelin effects (Fernández-Fernández *et al.* 2005c). In contrast, in prepubertal females (present experiments), ovariectomy, as well as treatment with a selective antiestrogen, were followed by an increase in the pituitary sensitivity to ghrelin action. Such age difference may be consequence of the different functionality of the prepubertal and adult ovary. Alternatively, changes in pituitary function along lifespan may also account for such a divergence. Indeed, prepubertal and adult pituitary differs in the morphological features of LH-secreting cells (Bello-Pineda *et al.* 1999), as well as in relative expression levels of ghrelin and GHS-R mRNAs. On the latter, expression of both genes at the pituitary is significantly higher in prepubertal than in adult male and female rats (Kamegai *et al.* 1999, Torsello *et al.* 2003).

The mechanism(s) involved in the stimulatory effect of ghrelin at the pituitary level remains unknown. In this context, a key issue is whether ghrelin action is primarily conducted directly on gonadotrops or, alternatively, via other pituitary cells, which may conduct paracrine actions upon gonadotrops. The cellular localization of ghrelin receptor at the pituitary has not been clearly established, and analyses on the regulation of GHS receptors at this site have been solely conducted in whole pituitary tissue (McKee *et al.* 1997, Kamegai *et al.* 1999, Kineman *et al.* 1999, Horikawa *et al.* 2000, Katayama *et al.* 2000, Nass *et al.* 2000). If GHS receptors are absent in gonadotrops, it should be assumed that the effects of ghrelin on LH secretion are exerted via a paracrine action, using one or some of the plethora of signals involved in intercellular communication at the pituitary (Schwartz 2000). Indeed, we have proposed previously the possibility that NO could mediate the

effect of ghrelin on LH secretion, since NO conducts direct stimulatory actions on LH and FSH secretion through a calcium-dependent, cGMP-independent mechanism (Pinilla *et al.* 1998), and it appears to mediate other relevant ghrelin actions, such as those on GH release (Pinilla *et al.* 2003), vascular relaxation (Shimizu *et al.* 2003), and food intake (Gaskin *et al.* 2003). In addition, hypothalamic NO synthases are increased by ghrelin (Gaskin *et al.* 2003). Present experiments support this possibility, since blockade of NO synthase with L-NAME abolished the stimulatory action of ghrelin on LH secretion. However, the cellular source of NO required for the expression of ghrelin action remains to be elucidated.

Our previous data demonstrated that ghrelin blunted LH responses to GnRH in adult rats regardless of the stage of cycle (Fernández-Fernández *et al.* 2005c). Present experiments showed, in contrast, that in intact and ovariectomized prepubertal rats ghrelin potentiated the stimulatory effect of GnRH on LH secretion. In addition, our data also indicate that this effect is augmented after ovariectomy, which suggests that ovarian inputs are involved in the modulation of ghrelin effects upon the responsiveness of LH to GnRH; an event already described in adult females (Fernández-Fernández *et al.* 2005c). The mechanism(s) whereby ghrelin is capable to modulate the stimulatory effect of GnRH on LH secretion is unknown. It might be possible that ghrelin participates in the tuning of GnRH binding to its own receptor or, alternatively, the intracellular actions of GnRH, as described previously for other peptides involved in the control of GnRH action, such as NPY (Parker *et al.* 1991, Evans 1999), galanin (Parker *et al.* 1991), or endothelins (Kauyicska *et al.* 1991). Considering that the intracellular signaling of GnRH and ghrelin is mediated by the same family of G proteins (Naor *et al.* 2000, Liu *et al.* 2002, Kojima & Kangawa 2005), a crosstalk between both signals is plausible, a possibility that is presently under evaluation at our laboratory.

In conclusion, present experiments evidenced a direct stimulatory effect of ghrelin on pituitary LH secretion in prepubertal female rats. This stimulatory effect was increased in the absence of estrogenic inputs and was mediated by NO. Overall, these data reinforce the concept that ghrelin participates in the control of pituitary hormones other than GH (van der Lely *et al.* 2004, Tena-Sempere *et al.* 2004), and suggest the involvement of ovarian signals in the modulation of the effects of ghrelin on LH secretion at the pituitary level.

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