Role of ghrelin in reproduction

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Abstract

Ghrelin, the endogenous ligand of GH secretagogue receptor type 1a, has emerged as a pleiotropic modulator of diverse biological functions, including energy homeostasis and, lately reproduction. Here, we review recent reports evaluating the reproductive effects and sites of action of ghrelin, with particular emphasis regarding its role as a molecule integrating reproductive function and energy status. Data gleaned from rodent studies clearly show that besides having direct gonadal effects, ghrelin may participate in the regulation of gonadotropin secretion and it may influence the timing of puberty. In addition, experimental data showing that ghrelin and/or its receptor are expressed in normal human ovary and testis as well as in human ovarian and testicular tumors raise the possibility that the ghrelin system may be involved in the control of cell proliferation in these tumors. We propose that ghrelin either acting as an endocrine and/or paracrine signal may play a major role in the endocrine network that integrates energy balance and reproduction.

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Introduction: historical remarks

Major attention on ghrelin started in December 1999, when the endogenous ligand for the growth hormone secretagogue receptor (GHS-R) type 1a was reported (Kojima *et al.* 1999). The ligand was named ghrelin from the Proto-Indo-European word of 'ghre', which means grow, and 'relín' as it had GH-releasing activities. Ghrelin is a 28 amino acid peptide with a fatty acid chain modification on the N-terminal third amino acid. Interestingly, at the same time, a stomach-derived mRNA sequence was identified as coding for a protein with sequence similarities to motilin and named motilin-related peptide m46 (Tomasetto *et al.* 2000). Structural analysis, aimed at identifying the amino acid sequence of ghrelin, identified a discrepancy between the observed and calculated molecular weight and pointed to the presence of a post-translational modification. Indeed, the hydroxyl group of the third N-terminal amino acid serine residue was found to be esterified by an *n*-octanoic acid (Kojima *et al.* 1999). No other naturally occurring peptide has been previously shown to have this acyl group as a post-translational modification. A spliced variant of ghrelin with 27 amino acids, missing

the 14th amino acid (glutamine), was also identified. Later, biologically active analogues of ghrelin were described in much smaller amounts with acyl chains of 10 or 11 C atoms or carbon unsaturations (Hosoda *et al.* 2000b). The *n*-octanoyl group at the Ser³ of the ghrelin molecule seems to be essential for some of the hormone's bioactivity, including GH release and orexi-genic effect. Unacylated (desoctanoyl or desacyl) ghrelin circulates in far greater concentrations than the acylated form and does not displace ghrelin from its hypothalamic and pituitary binding sites (Hosoda *et al.* 2000a) and is unable to stimulate GH release *in vivo* in rats and humans (Kojima *et al.* 1999, Broglio *et al.* 2003). Although in the beginning it was widely accepted that unacylated ghrelin (UAG) was devoid of any biological activity, increasing numbers of studies report different biological effects of UAG, which is now considered as a peptide with specific and wide ranging actions in different tissues (Cassoni *et al.* 2001, Bedendi *et al.* 2003, Nanzer *et al.* 2004, Thompson *et al.* 2004).

This review will more closely address the role of ghrelin in human and rodent reproduction, rather than its role as metabolic hormone, by focusing on its endocrine

and/or paracrine effects in the regulation of the hypothamic–pituitary–gonadal axis.

Ghrelin system in the testis

One of the reproductive tissues where expression and/or function of ghrelin were first evaluated was the testis. The possibility of direct gonadal actions of ghrelin was conceivable given the documented effects of other peripheral regulators of energy balance, such as leptin, in the male gonad (Tena-Sempere & Barreiro 2002). Indeed, in the last three years, a wealth of evidence has been gathered suggesting that ghrelin and its functional receptor, the GHS-R1a, are expressed in mammalian testis, where ghrelin might conduct specific regulatory actions.

Rodent data

The initial evidence for testicular expression of the ghrelin gene came from a study by Tanaka *et al.* (2001), who identified a testis-specific ghrelin gene derived transcript in the mouse. This ghrelin transcript was composed of a distinct 68-bp sequence at the 5'-end, followed by a 252-bp sequence corresponding to exons 4 and 5 of the mouse ghrelin gene (Tanaka *et al.* 2001). Notably, expression of 'full-length' ghrelin mRNA in the mouse testis was not detected in that study, using Northern hybridization.

In parallel, our research group initiated the analysis of the pattern of expression of ghrelin and its cognate receptor (GHS-R1a) in the rat testis, by a combination of molecular and immunological approaches. Thus, testicular ghrelin gene expression was demonstrated by semiquantitative RT-PCR throughout postnatal development, and ghrelin peptide was selectively detected in rat Leydig cells at advanced stages of maturation, regardless of their fetal or adult origin (Barreiro *et al.* 2002, Tena-Sempere *et al.* 2002). Accordingly, ghrelin expression became undetectable after selective elimination of mature Leydig cells by administration of the cytotoxic compound ethylene dimethane sulfonate (EDS; Barreiro *et al.* 2002, Tena-Sempere *et al.* 2002). Analyses of its regulation by hormonal signals revealed that ghrelin expression in the testis is not apparently modulated by follicle-stimulating hormone (FSH), GH, thyroid hormones and glucocorticoids, but it is, at least partially, under the control of pituitary luteinizing hormone (LH; Barreiro *et al.* 2002). This is in good agreement with the fact that testicular LH/CG receptors are solely expressed in Leydig cells (Tena-Sempere & Huhtaniemi 2003).

In addition to the ligand, expression of the putative ghrelin receptor was demonstrated by a combination of semiquantitative RT-PCR and *in situ* hybridization in rat testis (Tena-Sempere *et al.* 2002, Barreiro *et al.* 2003).

Notably, net testicular expression of *GHS-R* gene was detected at rather constant relative levels throughout postnatal development in the rat. In contrast, isoform-specific GHS-R1a mRNA was undetectable at prepubertal stages whereas it sharply increased thereafter, with peak values in adulthood (Barreiro *et al.* 2003). This observation suggests that during pubertal development a shift in the pattern of splicing of the *GHS-R* gene takes place in rat testis that favours expression of the biologically active type 1a form of the receptor, which might imply considerable changes in net ghrelin sensitivity with sexual maturation. Location analyses in the rat testis by means of immunohistochemistry and *in situ* hybridization revealed a scattered pattern of distribution of GHS-R1a, with specific expression in somatic Sertoli and Leydig cells, and eventually in germ cells (Barreiro *et al.* 2003). As was demonstrated for the ligand, testicular expression of *GHS-R* gene appeared under hormonal regulation: GHS-R1a mRNA levels were stimulated, within a range of doses, by ghrelin and pituitary FSH. Thus, not only ghrelin expression, but also testicular sensitivity to ghrelin is likely regulated by hormonal (homologous and heterologous) signals, which is highly suggestive of a finely tuned, direct action of ghrelin in the control of testicular function.

Besides expression analyses, direct biological actions of ghrelin in the rat testis have been demonstrated in our laboratory using a combination of *in vivo* and *in vitro* approaches. First, ghrelin was proven to significantly inhibit, in a dose-dependent manner, stimulated testosterone secretion. The inhibitory effect of ghrelin upon testosterone secretion was associated with a significant decrease in human choriogonadotropin (hCG)-stimulated levels of the mRNAs encoding several key factors in the steroidogenic route, such as steroid acute regulatory protein, and the enzymes P450 side-chain cleavage, 3 β -hydroxyl steroid dehydrogenase (HSD) and testis-specific 17 β -HSD type III. The fact that ghrelin equally decreased hCG- and cAMP-induced testosterone secretion indicates that this inhibitory action must take place in a step beyond cAMP formation (Tena-Sempere *et al.* 2002). Considering that the major stimulant for testicular expression of ghrelin is pituitary LH, it is tempting to speculate that ghrelin might operate as a local regulator in the fine-tuning of the steroidogenic actions of LH, which might participate in the auto-limitation of testicular testosterone response to gonadotropic stimulation (Barreiro *et al.* 2002). Alternatively, such an inhibitory effect upon testosterone secretion might be conducted by the systemic, gut-derived ghrelin, whose plasma levels are inversely correlated with body mass index (Dornonville *et al.* 2005). Thereby, elevated ghrelin levels (as those observed in energy insufficiency) might contribute to the suppression of male reproductive axis in situations of negative energy balance, such as starvation.

In addition to its steroidogenic effects, ghrelin might also directly regulate seminiferous tubule functions, as expression of GHS-R1a was demonstrated in the tubular compartment of the testis (Barreiro *et al.* 2003). Indeed, we have recently demonstrated the ability of ghrelin to inhibit expression of the gene encoding stem cell factor (SCF), both after intratesticular injection *in vivo* as well as following challenge of staged seminiferous tubule fragments in culture (Barreiro *et al.* 2004). Notably, SCF is a Sertoli cell product that has been identified as the major paracrine stimulator of germ cell development, acting as a survival factor for spermatogonia, spermatocytes and spermatids in the adult rat seminiferous epithelium (Hakovirta *et al.* 1999, Yan *et al.* 2000b). In addition, testicular SCF has been involved also in Leydig cell development and survival (Yan *et al.* 2000a). Thus, the actions of ghrelin upon tubular SCF mRNA expression may have implications not only in the control of spermatogenesis but also upon Leydig cell proliferation. In fact, using *in vivo* models of intratesticular injection, we have recently demonstrated that ghrelin is able to inhibit the proliferative rate of immature Leydig cells both during puberty development and after selective ablation of pre-existing mature Leydig cells by administration of EDS (Barreiro *et al.* 2004). These observations highlight the potential complexity of ghrelin actions in the testis, where this peptide might operate as novel regulator of Leydig cell development. Moreover, besides its impact on testicular function, our current data open up the possibility that ghrelin may regulate additional biological systems through modulation of SCF gene expression.

Human data

Although most data on the expression and biological actions of ghrelin in the male gonad has been obtained in the rat, fragmentary evidence indicates that ghrelin and its functional receptor are also expressed in the human testis. In fact, initial screening analyses of ghrelin mRNA expression in human tissues provided evidence of expression of ghrelin gene in the testis (Gnanapavan *et al.* 2002); a finding that was confirmed by our group (Gaytan *et al.* 2004). Thereafter, the pattern of testicular expression of ghrelin in the human was further characterized. Immunohistochemical analyses detected that, as is the case in the rat, ghrelin peptide is strongly expressed in interstitial mature Leydig cells. However, a specific feature of testicular expression of ghrelin in the human is the presence of this peptide, albeit at low levels, also in Sertoli cells (Barreiro *et al.* 2003).

Besides the ligand, GHS-R1a expression in the human testis was also detected by means of molecular and immunohistochemical techniques. Thus, the presence of GHS-R1a was demonstrated, at the mRNA and peptide levels, in human testis tissue (Gaytan *et al.* 2004). This is in good agreement with previous data showing the

presence of GHS binding sites in human gonads (Papotti *et al.* 2000). As was the case in the rat, testicular expression of GHS-R1a in the human showed a scattered pattern of distribution, with specific immunostaining being detected in somatic Sertoli and Leydig cells, as well as in germ cells, mainly in pachytene spermatocytes (Gaytan *et al.* 2004).

Ghrelin system in the ovary

Rodent data

Data gleaned recently have conclusively shown the expression of ghrelin (at the mRNA and peptide levels) in the adult rat ovary. To ascertain the physiological regulation of the ghrelin gene expression in the rat ovary, assessment of relative mRNA levels of this message was conducted through the different stages of the estrous cycle (Caminos *et al.* 2003). Interestingly, despite persistent expression of the signal at all stages tested, ghrelin mRNA levels significantly varied depending on the phase of the cycle, with the lowest expression levels in proestrus and maximum values in the diestrus (day 1) phase. Such a cyclic profile of expression, with peak levels in the luteal stages, is highly suggestive of predominant expression of ghrelin in the corpora lutea (CL) of the current cycle. Thus, ghrelin mRNA level reached their highest value when the CL entered into its functional phase and remained lower during CL formation and regression. This contention was further substantiated by immunohistochemical analyses, which showed intense and specific ghrelin immunoreactivity in the cytoplasm of steroidogenic luteal cells (Caminos *et al.* 2003). In addition, non-apoptotic cells in regressing CL from previous cycles and, to a lesser extent, cells from the interstitial gland showed detectable ghrelin immunostaining (Caminos *et al.* 2003). The profile of ghrelin expression in the CL was roughly coincidental with its peak in functional activity and paralleled the pattern of progesterone secretion (Duggal *et al.* 2002, Caminos *et al.* 2003), which is suggestive of a potential functional role of ghrelin in the regulation of luteal development and/or function in the rat. Nevertheless, whether ghrelin is actually involved in steroidogenesis, angiogenesis, tissue remodeling, and growth of the CL remains to be established. Overall, these data provide evidence that during the estrous cycle the expression of the gene encoding ghrelin undergoes dynamic changes that result in predominant expression of the peptide in the CL.

In keeping with the functional and morphological data indicative of a predominant expression of ghrelin in the CL within the cyclic ovary, blockade of the preovulatory surge of gonadotropins and subsequent ovulation by means of administration of a potent gonadotropin-releasing hormone (GnRH) antagonist significantly disturbed the cyclic profile of ovarian ghrelin mRNA

expression (Nekola & Coy 1985, Caminos *et al.* 2003). Indeed, ghrelin mRNA levels in ovaries from rats treated with a single dose of GnRH antagonist persistently remained at values similar to those in the proestrous stage and were significantly lower than those in paired diestrous d 1 and diestrous d 2 cyclic ovaries (Caminos *et al.* 2003). It is likely that prevention of ovulation which, in turn, blocks formation of the new CL, accounted for the decrease in ghrelin mRNA expression in ovaries from GnRH antagonist-treated rats (Nekola & Coy 1985, Caminos *et al.* 2003). Additionally, the reduction of circulating LH levels after GnRH antagonist treatment may cause the decrease in ovarian ghrelin mRNA levels. In this sense, we have recently provided evidence for a direct stimulatory role of LH/CG in the control of testicular ghrelin gene expression in interstitial Leydig cells, i.e. the major steroidogenic cell type of the testis. Whether an analogous phenomenon also operates in the ovary awaits further investigation.

In addition, ovarian ghrelin gene expression is markedly influenced by pregnancy. Expression levels of the messenger encoding ghrelin were higher in early pregnancy, and a significant decrease was detected during the later part of gestation (Caminos *et al.* 2003). In the rat, extension of the luteal function, which, in turn, acts to maintain pregnancy during the first week of gestation, is modulated by LH and prolactin (PRL; Freeman *et al.* 2000). However, gestation is maintained during the latter half of pregnancy in the rat by placental production of lactogen and androgens, and by estrogen and progesterone produced by CL (Ben Zimra *et al.* 2002). Most studies on the biochemistry and structure of the CL suggest that during late pregnancy the CL of the rat is at the very early stages of structural regression, with no changes at the morphological level, but with changes at the molecular level (functional regression). Overall, these data suggest that ghrelin is maximally expressed in functional CL from both current cycle and gestation, with declining expression levels along functional regression of CL. Nevertheless, detailed analyses of the pattern of ghrelin immunoreactivity in rat ovary throughout pregnancy are needed to fully substantiate this hypothesis. In addition, a recent report showed a specific ghrelin immunostaining in the labyrinth trophoblast cells of rat placenta at term (21 days p.c) with no positive signal in other placental cell types (Gualillo *et al.* 2001). A time-course study of ghrelin mRNA levels in rat placenta during pregnancy performed by semi-quantitative RT-PCR demonstrated a characteristic profile of expression being practically undetectable during early pregnancy, with a sharp peak at day 16 and still a quite degree of expression at term. Thus, it can be postulated that LH (and PRL) regulate the expression of ghrelin in the ovary during early pregnancy, whereas placental lactogen could play a pivotal role in the regulation of placental ghrelin mRNA expression during the latter half of pregnancy.

Human data

The presence and cellular location of ghrelin and its functional receptor, namely the type 1a GHS-R in the cyclic human ovary, have been reported using immunohistochemistry with polyclonal antibodies (Gaytan *et al.* 2003). Strong ghrelin immunostaining was demonstrated in ovarian hilus interstitial cells. By contrast, ghrelin signal was not detected in ovarian follicles at any developmental stage, nor was it present in newly formed CL in very early development. However, specific ghrelin immunoreactivity was clearly observed in young and mature CL, whereas expression of the peptide disappeared in regressing luteal tissue. Ovarian expression of GHS-R1a protein showed a wider pattern of tissue distribution, with detectable specific signal in oocytes as well as somatic follicular cells, luteal cells from young, mature, old, and regressing CL, and interstitial hilus cells. Of particular note, follicular GHS-R1a peptide expression paralleled follicle development with stronger immunostaining in granulosa and theca layers of healthy antral follicles. The presence of both components (ligand and receptor) of the ghrelin signaling system within the human ovary opens up the possibility of a potential regulatory role of this novel molecule in ovarian function under physiological and pathophysiological conditions. To this end, expression of GHS-R1a was analyzed by immunohistochemistry in a panel of normal, metaplastic, and neoplastic tissues (Gaytan *et al.* 2005). Uniform GHS-R1a immunostaining was detected throughout the ovarian surface epithelium. Likewise, ciliated cells within the fallopian tube epithelium showed strong GHS-R1a expression. By contrast, other celomic derivatives, such as endometrium and endocervix, were negative for GHS-R1a immunoreactivity. In keeping with data from normal tissues, inclusion cysts from the surface epithelium expressed GHS-R1a. Similarly, benign serous tumors resembling fallopian tube epithelium were also positive, whereas serous cystadenocarcinomas showed GHS-R1a expression only in highly differentiated specimens. By contrast, other neoplasms, such as mucinous cystadenomas and cystadenocarcinomas, endometrioid tumors, clear cell carcinomas, and Brenner tumors, did not express GHS-R1a. This pattern of expression is in agreement with the proposed role for ghrelin in the autocrine/paracrine control of cell proliferation and cancer. Proliferative actions of ghrelin (or its synthetic analogs, the GHSs) are observed in prostate (PC-3) and thyroid (anaplastic carcinoma; ARO) cancer cell lines as well as the cardiomyocyte H9c2 cell line and cultured adrenocortical cells (Cassoni *et al.* 2000, Pettersson *et al.* 2002, Andreis *et al.* 2003, Jeffery *et al.* 2003). Conversely, anti-proliferative effects of ghrelin/GHSs are observed in several thyroid (papillary carcinoma; NPA, follicular carcinoma; WRO), lung (CALU-1), and breast (MCF7, T47D, MDA-MB231) cancer cell lines (Cassoni *et al.* 2000, 2001, Ghe *et al.*

2002) as well as in immature Leydig cells of the testis (Barreiro *et al.* 2004). It remains to be proved whether the ovarian surface epithelium and related tumors are potential targets for systemic or locally produced ghrelin because they express the functional type 1a GHS-R. Considering the relevant role of the ovarian surface epithelium in key physiological events (such as ovulation) and neoplastic transformation of the ovary, the potential actions of ghrelin in those phenomena merit further investigation.

Systemic actions of ghrelin upon the reproductive axis

The reproductive facets of ghrelin are not apparently restricted to its expression and/or direct actions at the ovary and testis, and extragonadal actions of ghrelin within the reproductive axis have also been documented. However, so far the available data in this area are still fragmentary. In principle, considering the proposed role of ghrelin as peripheral signal for energy insufficiency (Zigman & Elmquist 2003), and the proven reproductive effects of other known regulators of energy balance, such as leptin, it appears feasible that circulating ghrelin might contribute to the functional control of the reproductive axis and its integration with energy balance.

Rodent data

Given their pivotal role in the hormonal control of reproduction (Tena-Sempere & Huhtaniemi 2003), the effects of ghrelin upon gonadotropin and PRL secretion have been explored in a limited number of models and animals species. Initial data evidenced that central administration of ghrelin suppressed pulsatile LH secretion in ovariectomized female rats (Furuta *et al.* 2001). In good agreement, intracerebroventricular administration of ghrelin (3 nmol/rat) evoked a significant inhibition of LH secretion in cyclic female rats throughout the estrous cycle (proestrus afternoon, estrus, metestrus), as well as in ovariectomized females (Fernandez-Fernandez *et al.* 2006). Likewise, GnRH secretion by hypothalamic fragments from ovariectomized females was significantly inhibited by ghrelin (Fernandez-Fernandez *et al.* 2006). In contrast, ghrelin dose-dependently stimulated basal LH and FSH secretion by pituitary tissue *in vitro*; a phenomenon that was proven dependent on the phase of estrous cycle, as it was neither detected at estrus nor observed after ovariectomy (Fernandez-Fernandez *et al.* 2006). Conversely, GnRH-stimulated LH secretion *in vitro* was persistently inhibited by ghrelin regardless of the stage of the cycle, whereas stimulated FSH secretion was only inhibited by ghrelin at estrus. In addition, cyclic fluctuations in mRNA levels of GHS-R 1a, i.e. the functional ghrelin receptor, were observed in the

pituitary, with low values at estrus and metestrus (Fernandez-Fernandez *et al.* 2006). In summary, these data illustrate a complex mode of action of ghrelin upon the gonadotropic axis, with predominant inhibitory effects at central (hypothalamic) levels and upon GnRH-induced gonadotropin secretion, but direct stimulatory actions on basal LH and FSH secretion. In this regard, our findings indicate that daily injection of ghrelin or UAG throughout puberty in male rats similarly decreased LH concentrations (Martini *et al.* 2006). Moreover, acute injection of ghrelin induced a transient reduction levels and partially delayed balanopreputial separation (Fernandez-Fernandez *et al.* 2005, Martini *et al.* 2006; Fig. 1). Likewise, chronic infusion of ghrelin

Figure 1 Ghrelin and UAG – puberty in males. Repeated administration of ghrelin or UAG to pubertal male rats partially alters normal puberty onset. Repeated injection of acylghrelin or UAG (1 nmol/12 h) between postnatal days 34–43 significantly decreased serum LH (A) and testosterone (B) levels and partially disrupted normal timing of puberty onset (C), as estimated by the presence of balanopreputial separation (BPS) at d-43. Values are given as mean $\pm$ S.E.M. For vehicle treated animals, groups with different superscript letters were statistically different ($P < 0.05$). ** $P < 0.01$ vs corresponding vehicle treated animals. Adapted from Fernandez-Fernandez *et al.* (2005) and Martini *et al.* (2006).

or UAG to adult males resulted in significant decreases in circulating LH and FSH levels in freely moving males, an effect that was fully mimicked by administration of UAG (Martini *et al.* 2006; Fig. 2). Yet in contrast to ghrelin, UAG failed to modify GH secretion. Finally, injection of ghrelin moderately, but significantly, reduced the duration of LH secretory responses to the potent gonadotropin secretagogue kisspeptin-10, whereas ghrelin infusion in a model of chronic elevation of serum gonadotropin levels (the transgenic growth retarded male rat) evoked a significant reduction of LH concentrations (Martini *et al.* 2006). Altogether these data further substantiate the inhibitory effect of ghrelin on basal and stimulated LH secretion in a wide array of experimental conditions. Moreover, these data show the ability of UAG, originally considered an inert form of the molecule, to mimic the actions of acylated ghrelin on LH release. These observations reinforce the contention that ghrelin, as putative signal for energy insufficiency, may operate as negative modifier of male puberty and LH secretion, an effect that might be, at least partially, conducted through a GH secretagogue receptor type 1a-independent mechanism. The mechanisms for such an effect might involve the inhibition of LH secretion at the hypothalamic–pituitary unit and/or direct inhibitory effects on testicular testosterone secretion.

Besides its neuroendocrine effects upon gonadotropic and lactotropic axes in the rat, ghrelin expression has been also detected in rat placenta (Gualillo *et al.* 2001). In

addition, ghrelin levels in uterine fluid have been proven to dramatically increase during fasting in mice, and ghrelin has been recently reported to inhibit the development of mouse preimplantation embryos *in vitro* (Kawamura *et al.* 2003). In good agreement, we have recently observed that chronic ghrelin treatment during the first half of pregnancy in the rat induced a significant reduction in the litter size (Fernandez-Fernandez *et al.* 2005). Overall, it is tempting to propose that ghrelin may operate as key signal for energy insufficiency during early stages of gestation, acting as inhibitory factor in early embryo development in order to avoid the excessive metabolic drain linked to pregnancy and lactation in situations of malnutrition (Kawamura *et al.* 2003).

Human data

The potential effects of ghrelin in the control of the reproductive system in humans (and primates) have been very scarcely evaluated. Only recently, the original observations of an inhibitory role of ghrelin in the control of LH secretion in rodents (Furuta *et al.* 2001, Fernandez-Fernandez *et al.* 2004) have been corroborated by experiments conducted in ovariectomized rhesus monkeys, after detailed analyses of the pulsatile pattern of LH release after infusion of ghrelin (Vulliamoz *et al.* 2004). In humans, however, although stimulatory responses in terms of PRL secretion have been repeatedly reported after acute administration of the peptide, specific effects of ghrelin upon LH secretion have not been demonstrated (van der Lely *et al.* 2004). Yet, it remains possible that more detailed analyses, involving precise assessment of LH pulsatility after ghrelin administration, might unravel a subtle regulatory role of ghrelin in the control of gonadotropin secretion in humans. Of note, androgens have been recently shown as independent modulators of ghrelin levels in male and female humans (Pagotto *et al.* 2002, 2003, Gambineri *et al.* 2003). The polycystic ovary syndrome (PCOS), is a condition characterized by hyperandrogenism, chronic anovulation, insulin resistance, and frequently associated with obesity (Micic *et al.* 2003). Pagotto *et al.* (2002) have reported that obese women with PCOS have even lower ghrelin levels than expected on the basis of their obese phenotype and a strong negative correlation between ghrelin and circulating androgen parameters, particularly androstendione, was also described (Pagotto *et al.* 2002). Moreover, a long-term treatment with the anti-androgen agent, flutamide, in PCOS women significantly increased ghrelin levels (Gambineri *et al.* 2003). The same group also found that circulating ghrelin levels were significantly decreased in obese hypogonadal men compared to their overweight matched controls and a positive correlation between ghrelin and total and free testosterone concentrations was described in this study (Pagotto *et al.* 2003). Likewise, a long-term replacement testosterone therapy

Figure 2 Ghrelin and UAG–Serum gonadotropin levels in males. Chronic i.v. administration of ghrelin or UAG (1 nmol/12 h) for seven days in adult male rats significantly decreased serum LH (A) and FSH (B) levels. Values are given as mean $\pm$ S.E.M. * $P < 0.05$ and ** $P < 0.01$ vs corresponding vehicle treated animals. Adapted from Martini *et al.* (2006).

in hypogonadal patients restored circulating ghrelin levels to those found in control normal subjects. Thus, it appears feasible that ghrelin and sex steroids reciprocally interact (ghrelin modulates androgen secretion – rat data-; androgen modulates ghrelin secretion–human data-) for the integrated control of reproduction and energy balance.

As was the case in the rat, ghrelin expression has been also detected in human placenta (Gualillo *et al.* 2001), and ghrelin has been demonstrated in human fetal circulation (Cortelazzi *et al.* 2003). However, the role, if any, of placental and fetal ghrelin in the regulation of gestational growth and metabolism remains to be fully elucidated. In addition, expression of ghrelin has been observed in non-pregnant and decidualized endometrium, and ghrelin has been involved as paracrine/autocrine regulator of decidualization of human endometrial stromal cells and, tentatively, in the cross-talk

between endometrium and embryo during implantation (Tanaka *et al.* 2003). Whether ghrelin, as suggested for laboratory rodents, may function as putative modifier of human gestation in situations of energy deficit remains to be elucidated.

Unsolved questions: concluding remarks

Our knowledge on the potential actions of ghrelin on the mammalian gonads has enlarged considerably in the last few years, yet there are still many open questions concerning the physiological contribution of ghrelin in the control of gonadal function. For instance, whereas potential inhibitory effects of ghrelin upon testicular steroid secretion have been evidenced using *in vitro* models (Fig. 3; Tena-Sempere *et al.* 2002), elucidation of the impact of persistent hyperghrelinemia upon circulating levels of androgens and estrogens *in vivo* awaits to be

Figure 3 Schematic model for the postulated reproductive actions of ghrelin. In the upper panel, the potential SYSTEMIC actions of gut-derived circulating ghrelin at different levels of the hypothalamic–pituitary-axis are depicted. In addition, LOCAL expression of ghrelin is detected in the human and rat gonads. In the lower panel the proposed direct actions of ghrelin in the testis and ovary are summarized. A question mark (?) indicates that the effect was not studied.

elucidated. Likewise, ovarian and testicular actions of ghrelin other than the control of the steroidogenic function are anticipated (Barreiro *et al.* 2004); yet, they remain to be studied in detail (Fig. 3). In this sense, it will be interesting to test whether unacylated ghrelin, conventionally regarded as an inert form of the molecule but recently proven as biologically active in specific cellular contexts (van der Lely *et al.* 2004) might mimic and/or modulate the reported effect of acylated ghrelin upon cell proliferation and gene expression in ovary and testis. Finally, an interesting observation is that several testicular tumors were recently proven to express ghrelin and/or GHS-R1a (Gaytan *et al.* 2004). It is yet to be determined, however, whether ghrelin might conduct specific regulatory actions upon testicular tumor cell proliferation. To be noted, expression of ghrelin has been previously demonstrated in several endocrine tumors, such as pituitary-, stomach- and pancreas-derived tumors, i.e. tissues that normally express ghrelin (van der Lely *et al.* 2004), and proliferative or anti-proliferative effects of ghrelin have been variably reported depending on the tumor cell line and dose of ghrelin used (Jeffery *et al.* 2003).

Although testing of the effects of its acute administration has suggested a possible role of ghrelin in the control of the gonadotropic axis (Fig. 3), the impact of persistently elevated ghrelin levels upon sensitive reproductive parameters has not been addressed so far. The working hypothesis is that, given its role as a signal for energy insufficiency, chronic hyperghrelinemia (as expected in situations of negative energy balance) might be detrimental for reproductive function. Indeed, studies on the effects of repeated administration of ghrelin upon several reproductive parameters of the rat are currently in progress in our laboratory. Likewise, we are presently analyzing the primary sites and mechanisms of action whereby ghrelin regulates LH and PRL secretion. We anticipate that elucidation of the sites and mechanisms of action of ghrelin, together with the complete characterization of its effects, will help to define the physiological roles of ghrelin in the control of the reproductive system, and its contribution for the integration of energy balance and fertility. Noteworthy recent reports (Smith, unpublished observations) have indicated that marked impairment in gonadal function and fertility in the ob/ob mice can be reversed when crossed with the ghrelin KO mice, imply a major role of leptin and ghrelin in the control of gonadal function. Future studies, assessing the analysis of the putative reproductive effects of octanoylated- as well as unacylated-ghrelin, not only upon pituitary hormone secretion, but also in terms of sexual development and gestation are eagerly awaited.

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