The role of intragestational ghrelin on postnatal development and reproductive programming in mice

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Abstract

The purpose of this study was to evaluate the intragestational role of ghrelin in offspring development and reproductive programming in a mouse model of ghrelin imbalance during pregnancy. Female mice were injected with ghrelin (supraphysiological levels: 4 nmol/animal/day), antagonist (endogenous ghrelin inhibition with (D-Lys$_3$)GHRP-6, 6 nmol/animal/day) or vehicle (control = normal ghrelin levels) throughout the pregnancy. Parameters evaluated in litters were growth, physical, neurobiological and sexual development and, at adulthood, reproductive function. Litter size and initial weight did not vary between treatments. Male pups from dams treated with ghrelin showed higher body weight increase until adulthood (31.7 ± 0.8 vs control = 29.7 ± 0.7, $n = 11$–14 litters/treatment; $P < 0.05$). Postnatal physical and neurobiological development was not modified by treatments. The antagonist accelerated male puberty onset, evidenced as earlier testis descent and increased relative testicular weight (antagonist = 0.5 ± 0.0% vs ghrelin = 0.4 ± 0.0% and control = 0.4 ± 0.0%, $n = 5$–10 litters/treatment; $P < 0.05$). At adulthood, these males exhibited lower relative testicular weight and reduced sperm motility (63.9 ± 3.6% vs control = 70.9 ± 3.3 and ghrelin = 75.6 ± 3.0, $n = 13$–15 animals; $P < 0.05$), without changes in plasma testosterone or fertility. Female pups intragestationally exposed to the antagonist showed earlier vaginal opening (statistically significant only at Day 25) and higher ovarian volume (antagonist = 1085.7 ± 64.0 mm³ vs ghrelin = 663.3 ± 102.8 mm³ and control = 512.3 ± 116.4 mm³; $n = 4$–6 animals/treatment; $P < 0.05$), indicating earlier sexual maturation. At adulthood, these females and those exposed to ghrelin showed a tendency to higher percentages of embryo loss and/or foetal atrophy. In conclusion, ghrelin participates in reproductive foetal programming: alterations in ghrelin activity during pregnancy modified body weight increase and anticipated puberty onset, exerting (or tending to) negative effects on adult reproductive function.

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Introduction

Ghrelin (GHRL) is a 28 amino acid peptide that has been linked to reproductive physiology, particularly the modulation of the hypothalamic–hypophyseal–gonadal axis (Kojima *et al*. 1999, 2001, Barreiro *et al*. 2002, Kawamura *et al*. 2003, Fernández-Fernández *et al*. 2006, García *et al*. 2007, Zhang *et al*. 2007, Tena-Sempere 2008a). Because plasma GHRL concentrations dramatically increase during fasting or undernourishment (Kojima & Kangawa 2005), this peptide has been proposed as an inhibitory signal for reproductive physiology and behaviour during food scarcity periods (Schneider 2004, Fernández-Fernández *et al*. 2006, Tena-Sempere 2008a,b, Bertoldi *et al*. 2011).

A physiological increase of maternal and foetal GHRL levels has been registered during pregnancy in mammals (including humans) (Gualillo *et al*. 2001, Shibata *et al*. 2004, Fuglsang *et al*. 2005, Govoni *et al*. 2007, Harrison *et al*. 2007, Palik *et al*. 2007), which suggests that the peptide may play an important role in gestation. Furthermore, since an active GHRL receptor (GHS-R1a) has been detected in gametes, embryos, placenta, endometrium and fallopian tubes, it has been suggested that GHRL might be one of the numerous peptides that regulate embryo development and/or implantation (Barreiro *et al*. 2002, 2003, Caminos *et al*. 2003, Kawamura *et al*. 2003, Gaytan *et al*. 2004, 2005, Harrison *et al*. 2007, Zhang *et al*. 2007, Du *et al*. 2010, Steculorum & Bouret 2011). In a previous study using an *in vivo* mouse model of intragestational GHRL imbalance (the same one used in the present study), we confirmed that gestational GHRL plays a role in fertilisation, preimplantation embryo development and implantation.

Indeed, we found some detrimental effects not only with the administration of GHRL (supraphysiological levels) but also with the injection of the antagonist (endogenous ghrelin inhibition), supporting the proposition that GHRL (in 'adequate' concentrations or activity) has a physiological role in early gestational events (Luque *et al.* 2014).

A few studies also indicate a function of intragestational GHRL in foetal and/or postnatal development. We noticed that chronic food restriction for mouse dams (from Days 1 to 17 of pregnancy), which increases plasma GHRL, provoked developmental defects in the offspring, such as physical, neurobiological and reproductive maturation delay (Torres *et al.* 2017). It has been demonstrated that maternal GHRL easily crosses the placental blood barrier into the foetal circulation and amniotic fluid in rodents (Kawamura *et al.* 2003), and gestational food restriction leads to a decrease in offspring body weight at birth (Desai *et al.* 2005, Chanoine *et al.* 2006, Wang *et al.* 2007). However, two studies have reported that the administration of GHRL to pregnant rats from Day 14 to 15 until birth increased offspring body weight (Hayashida *et al.* 2002, Nakahara *et al.* 2006).

In a previous study using a GHRL analogue, hexarelin (200 µg/kg/day), during different stages of pregnancy in mice, we demonstrated that hexarelin, administered during the second or third week of pregnancy (but not during the first week), increased pup body weight gain until adulthood without modifying the initial weight (Luque *et al.* 2010). It is thus evident that differences between studies depend on the period of gestation in which GHRL rises (naturally or by its exogenous administration) and, probably, on its concentration.

Developmental programming can be defined as a response of mammalian organisms to a specific challenge during a critical development time window (in this case, gestation), which alters the trajectory of development with persistent effects on the offspring phenotype (Rabadan-Diehl & Nathanielsz 2013). Since the reproductive axis and its hormonal control are largely established during foetal life, they are a target for developmental programming (Chadio & Kotsampasi 2014).

Although many studies have evaluated the possible effects of intragestational under/over-nutrition on reproductive foetal programming (with negative effects reported at both ends of the nutritional spectrum) (Castellano *et al.* 2011, Chadio & Kotsampasi 2014, Zambrano *et al.* 2014), few investigators have explored the effects of intragestational GHRL imbalances. In general, high doses of GHRL administered to pregnant rats (Hayashida *et al.* 2002, Nakahara *et al.* 2006) have induced results comparable to undernourishment protocols (evidenced by an increase in the dams' daily food intake), in which GHRL acts as a starvation signal. Therefore, the information obtained from these studies

about the physiological role of GHRL during pregnancy is limited. Moreover, to our knowledge, there is only one study of the effects of maternal GHRL deficiency (comparing homozygote with heterozygote dams) on reproductive foetal programming of female offspring (Martin *et al.* 2011).

Therefore, the purpose of this study was to evaluate, using a previously validated mouse *in vivo* approach (Luque *et al.* 2014), the intragestational role of GHRL on postnatal development, assessing physical, neurobiological and sexual parameters, as well as the reproductive function of the offspring at adulthood.

Since underweight, obesity and polycystic ovarian syndrome are associated with modifications in normal ghrelinaemia (Repaci *et al.* 2011, Goebel-Stengel *et al.* 2013), it is important to recognise the possible consequences of these imbalances in offspring development.

Materials and methods

Animals

Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the Medical School of the Universidad Nacional de Córdoba (UNC-RHCS 674/09). Its animal ethics committee approved the protocols used in this study (April 14, 2014).

We used adult (60–80 days; 30–35 g) female inbred *Albino Swiss* mice (N:NIH) maintained on a 14:10-h light:darkness regime at $23 \pm 2°C$, with access to water and food (Grupo Pilar-Gepsa, Cordoba, Argentina) *ad libitum*. It should be noted that our previous experiments established that the doses of GHRL or antagonist used in this study do not significantly modify the daily food intake or body weight (Luque *et al.* 2014). Nevertheless, results of food intake and dam body weight from this specific study are presented in 'Results' section.

Chemicals

GHRL (Innovagen, Lund, Sweden) and its antagonist, Ant: $(D-Lys_3)GHRP-6$ (Sigma-Aldrich), were dissolved in isotonic solution (0.9% ClNa solution) and administered by subcutaneous injection twice a day (with half of the daily dose in each injection, at 09:00h and 17:00h). Control animals received the vehicle in the same regimen. The GHRL doses used in this study were established based on its ability to increase growth hormone secretion from a dose–response curve previously performed and published by our group (Bertoldi *et al.* 2011). Concordantly, the selected dose of the antagonist was previously demonstrated to inhibit the effects of endogenous hyperghrelinaemia or exogenously administered GHRL, which makes this option an effective GHRL antagonist protocol (Bertoldi *et al.* 2011, Luque *et al.* 2014). Plasma GHRL results in dams are shown in the corresponding section. This parameter was evaluated by means of a specific ELISA kit (see below).

Experimental groups

From the day of copula confirmation (by observation of vaginal plug or spermatozoa in vaginal smears) and throughout pregnancy, female mice were subjected to one of the following treatments:

- Ghrelin (GHRL): 4 nmol/animal/day of GHRL dissolved in 0.2 mL of isotonic solution (i.e. treatment that induces supraphysiological levels of ghrelin).
- Antagonist (Ant): 6 nmol/animal/day of (D-Lys$_3$)GHRP-6 (GHRL antagonist) dissolved in 0.2 mL of isotonic solution (i.e. treatment that inhibits endogenous ghrelin actions).
- Control (C): 0.2 mL of isotonic solution/animal/day (i.e. physiological ghrelin levels).

Drugs were administered twice a day and the treatment stopped when delivery was confirmed. The offspring of these dams did not receive any further treatment.

Effects of intragestational GHRL *imbalance on physical, neurobiological and sexual development of the offspring*

Litter size and weight were recorded 24 h after birth (postnatal Day 1 = pnd 1). To avoid differences in body weight evolution during lactation due to differences in litter size, four male and four female pups per dam were randomly selected (the remaining pups in the litter were killed). The reduced litter (8 pups/dam) was weighed again and then once a week until adulthood, differentiating sexes (quantified as mean of the male or the female litter and finally expressed in 'Results' section as mean of litters/group). Results were expressed as initial weight, body weight evolution and body weight increase (absolute and as percentage).

Physical and neurobiological development was determined in the entire litter (i.e. without differentiating sexes) (following Santillán *et al.* 2010, Luque *et al.* 2010) as follows:

Physical development

Parameters evaluated were bilateral pinna detachment (unfolding of external ear, from pnd 1 to pnd 6), low incisors eruption (emergence from the gingival, from pnd 8 to pnd 13) and eyes opening (from pnd 10 to pnd 16) (Kihara *et al.* 2001, Bowers *et al.* 2004).

Neurobiological development

Three different reflex/behavioural tests were performed: (a) cliff avoidance: each animal was placed on a table edge with forepaws and nose over the edge (height 20 cm). Time required to complete backing and turning away from the edge was assessed. The number of animals with a successful response within 30 s was recorded. This parameter is considered a marker of maturity in sensory and motor functions associated with development and was evaluated at pnd 8 (Yoshida *et al.* 2000); (b) negative geotaxis: pups were placed in a head down position on a 45° inclined cardboard surface, and the time required to complete a 180° turn was measured. The number of mice with a successful response within 30 s was recorded.

This test reflects the function of the vestibular system and was evaluated on pnd 9 (Metz and Schwab 2004) and (c) surface righting reflex: each mouse was held on its back on a flat surface for 4 s and subsequently released. The number of animals that regained all four paws in contact with the surface within 2 s was recorded. This is a standard test for labyrinth function and body righting mechanism and was evaluated on pnd 10 (Vorhees *et al.* 1979).

Sexual development

Puberty onset was determined in males when both testicles descended and in females by day of vaginal opening. Additionally, oestrus cyclicity was evaluated from vaginal opening until adulthood by means of vaginal smears. The length of a cycle was measured from the first day of oestrus until the day before the next oestrus; only complete cycles were considered for statistics. A group of male and female pups were killed at pnd 19 and pnd 23 respectively, to weigh/measure their gonads. Testes were weighed using a digital scale. Ovarian volume was calculated by fixing the ovaries (in Bouin solution), including them in paraffin and performing multiple serial cuts to the gonad. Under an optic microscope, one section was randomly selected from the first eight sections, and measured; counting from that section onwards, every eighth section was also measured. Each evaluated section was measured using a scale incorporated into the microscope, assessing the perpendicular axes (a and b). According to the Cavalier principle, ovarian volume was calculated as follows: $V = \sum ai \times T \times 1/f$, with '$ai$' being the area of each section ($ai = (\pi/4)\,a.b$), 'T' the thickness of the sections (7 μm) and 'f' the fraction of the sections measured (1 from eight in our study).

Effects of intragestational GHRL *imbalance on the reproductive function of offspring in adulthood*

At adulthood (60 days), the reproductive function of the offspring was evaluated as follows:

Female evaluation

Females were allowed to mate with control males (without treatment) and killed to evaluate reproductive parameters at Day 18 of gestation as follows:

Number of corpora lutea   Both ovaries were dissected and the number of fresh corpora lutea/female was evaluated under a stereoscopic magnifying glass (Luque *et al.* 2014). In previous experiments performed in our laboratory, we observed, under controlled conditions, an equal number of corpora lutea and foetuses in more than 80% of the females. We therefore consider this parameter to be an acceptable sign of ovulation rate (Puechagut *et al.* 2012, Luque *et al.* 2014).

Atrophied foetuses   The uterus was extruded and the number and the weight of the foetuses were evaluated. Foetuses visually smaller than normal (two thirds or even smaller) were counted as 'atrophied' (Luque *et al.* 2014). This parameter was calculated not only for each female (number of atrophied foetuses/female) but also for each treatment, recording the

percentage of dams/treatment that exhibited some degree of foetal atrophy.

Embryo loss   Once the number of corpora lutea and foetuses (including atrophied ones) were evaluated at gestational Day 18, an index between these two variables was calculated by the following formula: $100 - (\text{number of foetuses} \times 100/\text{number of corpora lutea})$ (Luque *et al.* 2014). When more corpora lutea than foetuses were found, we considered that some alteration had occurred in the reproductive process; we named this event 'embryo loss' and expressed the variable as a percentage. Again, this parameter was calculated not only for each female (percentage of embryo loss/female) but also per treatment, i.e. recording the percentage of females/treatment that exhibited more corpora lutea than foetuses (independently of the index value).

Male evaluation

Fertility   At adulthood, male pups were allowed to mate with control females (without any treatment). At pregnancy Day 18, the females were killed to evaluate percentages of pregnancy and number and weight of foetuses. We also evaluated the proportion of embryo loss and atrophied foetuses as described above.

Plasma testosterone   Male pups were killed at adulthood by decapitation and blood was collected in tubes with EDTA and centrifuged at 120 **g** for 30 min. Plasma was stored at −20°C until processing. Testosterone concentration was determined with an in-house enzyme immunoassay using a polyclonal anti-testosterone antibody, testosterone standard and their corresponding horseradish peroxidase conjugates (testosterone R156/7, Department of Population Health and Reproduction, C. Munro, UC Davis, CA, USA). Briefly, flat bottom microtitre plates (Nunc Maxisorp, VWR, Mississauga, ON, Canada) were first coated with 50 µL of the anti-testosterone antibody diluted in coating buffer (50 mM bicarbonate buffer, pH 9.6; 1:10,500), covered with acetate sealers to prevent evaporation and incubated overnight at 4°C. After 16–24 h, plates were washed to remove any unbound antibody with 0.02% Tween 20 solution using a Bio-Tek ELx 405VR microplate washer (Bio-Tek Instruments). Immediately after washing, 50 µL of plasma samples, standards and controls were added in duplicates, followed by 50 µL of horseradish peroxidase conjugate diluted in EIA buffer (1:20,000). Plates were then covered and incubated at room temperature for 2 h. Following incubation, the plates were washed and blotted dry, and 100 µL of substrate solution (50 mM citrate, 1.6 mM hydrogen peroxide and 0.4 mM 2,20-azino-di-(3-ethylbenz-thiazoline sulfonic acid) diammonium salt, pH 4.0) were added to each well. Absorbance was measured at 405 nm using a microplate reader (Thermo Electron Corporation, USA). The assay sensitivity was 0.047 ng/mL. The intra-assay and inter-assay coefficients of variation were less than 10% and 15%, respectively. Cross-reactivity values were 5-α-dihydrotestosterone (57.4%), androstenedione (0.27%), androsterone (0.04%), cholesterol (0.03%) and <0.02% with all other steroids tested.

Sperm functional activity   Caudal epididymal spermatozoa were obtained as previously described (Fiol de Cuneo *et al.* 1994). Sperm concentration and motility were quantified in a Makler counting chamber (Makler 1980) (Sefi-Medical Instruments, Israel). Motility was expressed as a percentage of motile cells (progressive plus non-progressive gametes).

Sperm viability was assessed using the supravital stain, Hoechst 33258, as previously described (Kovács & Foote 1992) and evaluated with an epifluorescence microscope (Zeiss, Germany). Results were expressed as a percentage of viable cells.

The percentage of immature gametes was evaluated by quantifying the percentage of spermatozoa showing the bent morphology and/or a persistent cytoplasmic drop (Bedford *et al.* 1973, Cooper 1998, Cornwall *et al.* 1988).

The response to the hypoosmotic swelling test (PRO) was assessed as previously described (Fiol de Cuneo *et al.* 1994), incubating spermatozoa in hypoosmotic solution and quantifying those that reacted to this osmotic challenge 40 min later.

Acrosomal reaction was evaluated by the Coomassie blue technique (Larson & Miller 1999), and the results were expressed as the percentage of spermatozoa with intact acrosome.

Ghrelin determinations   Another group of dams was killed (at Day 8 of gestation) by decapitation and blood was collected in tubes with EDTA and centrifuged at 120 **g** for 30 min. Plasma was then collected and stored at −20°C until processing. Ghrelin concentrations were determined using a specific commercial ELISA kit (EK-031-31, Phoenix Pharmaceuticals Inc., CA, USA) following the manufacturer's instructions. This kit has a sensitivity of 0.12 ng/mL and a range of 0–100 ng/mL (linear range 0.12–1.26 ng/mL). The intra-assay and inter-assay coefficients of variation were less than 10% and 15%, respectively.

Statistical analysis

In general, results were expressed as mean ± s.e.m. and were analysed by one-way ANOVA with LSD Fisher as a *post hoc* comparison analysis. Results from parameters that were evaluated several times in the same litter (dam food intake and body weight, pup body weight, bilateral pinna detachment, lower incisor eruption, eyes opening, testis descent and vaginal opening) were evaluated by repeated-measures ANOVA (with evaluation day and treatment as fixed effects and litter as random effect), using LSD Fisher as *post hoc*. To apply these tests, the variance homogeneity and Gaussian distribution (by modified Shapiro–Wilk) were confirmed. Parameters expressed as a percentage (percentage of females with foetal atrophy/treatment and percentage of females with embryo loss/treatment) were analysed by chi-square test. In all cases, *P* values under 0.05 were considered statistically significant. Statistical analyses were performed with Infostat 2016p (Infostat Group, Facultad de Ciencias Agropecuarias – Universidad Nacional de Córdoba, Argentina).

Table 1 Food intake and body weight increase during pregnancy of dams treated with ghrelin or an antagonist.

Variable/day of pregnancy	Control ($n = 5$)	Ghrelin (4 nmol/animal/day) ($n = 5$)	Antagonist (6 nmol/animal/day) ($n = 6$)
Daily food intake (g)			
1	3.28 ± 0.34*	3.23 ± 0.41*	2.84 ± 0.32*
7	4.15 ± 0.37#	4.71 ± 0.57#	4.44 ± 0.66#
14	5.63 ± 0.53	5.02 ± 0.44	5.27 ± 0.52
18	5.75 ± 0.38	5.27 ± 1.56	6.33 ± 0.47
Body weight (g)			
1	25.83 ± 1.25°	24.88 ± 1.78°	23.92 ± 1.57°
7	26.61 ± 1.30°	26.31 ± 1.86°	24.52 ± 1.74°
16	38.75 ± 1.60@	35.57 ± 3.81@	34.62 ± 3.22@
19	46.19 ± 1.94	41.66 ± 7.15	45.72 ± 2.90

Ghrelin or its antagonist ((D-Lys3)GHRP-6) were administered (s.c.) during the whole pregnancy in two injections/day. Control dams were injected with vehicle in the same regimen (isotonic solution). Values are expressed as mean ± S.E.M. In parenthesis: number of dams evaluated. There are no differences between treatments. Differences between days, $P < 0.001$: *Day 1 vs Days 7, 14 and 18; #Day 7 vs Days 14 and 18; °Days 1 and 7 vs Days 16 and 19; @Day 16 vs Day 19.

Results

Dams were treated with GHRL, Ant or the vehicle throughout gestation. To corroborate previous results (Luque *et al.* 2014), we evaluated food intake during pregnancy in an initial group of dams. As expected, food intake increased along with pregnancy, but did not differ between groups (Table 1). Similar results were observed for the parameter 'body weight', which increased during pregnancy but was not different between treatments (Table 1). To biochemically validate our model, the ghrelin plasma levels in the dams were evaluated. Concentrations detected were C: 15.6 ± 2.2 ng/mL, GHRL: 42.0 ± 8.0 ng/mL and Ant: 11.8 ± 1.0 ng/mL ($n = 6$–7 dams/treatment; $P = 0.002$ GHRL vs C and Ant).

Effects of intragestational GHRL *imbalance on physical, neurobiological and sexual development of the offspring*

Neither litter size (C = 10.10 ± 1.02, GHRL = 10.17 ± 0.51 and Ant = 10.76 ± 0.38; $n = 10$–17 litters/treatment; $P > 0.05$) nor pup initial body weight (pnd 1) were modified by these treatments (C = 1.63 ± 0.06 g, GHRL = 1.66 ± 0.03 g and Ant = 1.59 ± 0.05 g, $n = 10$–17 litters/treatment; $P > 0.05$). Pup body weight was greater in pups from dams treated with GHRL, especially in males, in which differences remained significant from pnd 35 onward (Fig. 1). In fact, these pups also exhibited a significantly higher increase in body weight (calculated as final body weight − initial body weight: GHRL = 31.7 ± 0.8 vs C = 29.7 ± 0.7, $n = 11/14$ litters/treatment; $P = 0.035$). Female pups showed a similar tendency, but the values were statistically significant only on pnd 35 (Fig. 1).

Physical development, evaluated as bilateral pinna detachment, eruption of lower incisors and eyes opening, was not affected by treatments. As expected, the percentage of pups positive for each of these parameters increased with age (i.e. postnatal day)

(Fig. 2). Neurobiological maturation was not altered by treatments either (Table 2).

Male pups from dams treated with Ant showed earlier puberty onset, determined as time of testis descent. These differences were statistically significant at pnd 19, 20 and 21 (Fig. 3). Concordantly, these pups exhibited higher relative testicular weight compared to other
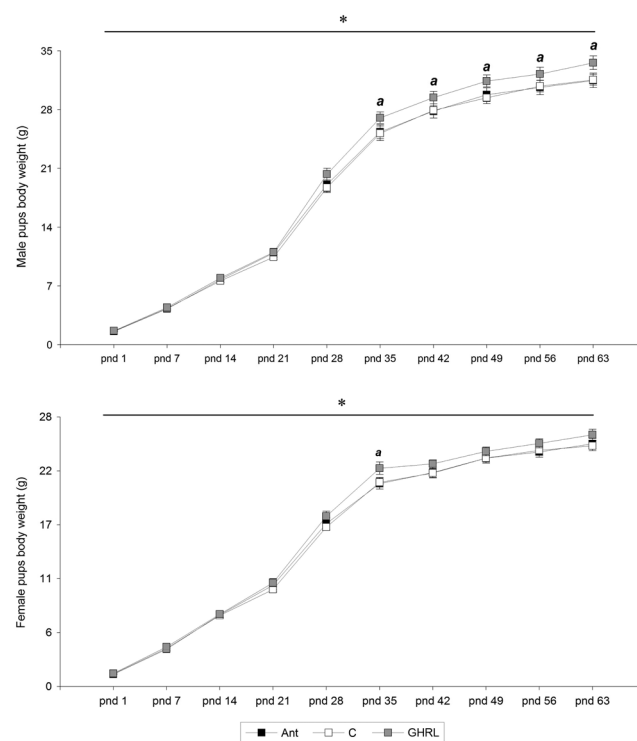

Figure 1 Body weight increase of male and female pups from birth (pnd 1 = postnatal Day 1) to adulthood (pnd 63). Dams were treated throughout pregnancy with ghrelin (GHRL: 4 nmol/animal/day), an antagonist (Ant: (D-Lys3)GHRP-6; 6 nmol/animal/day) or vehicle (C: isotonic solution). Values are expressed as mean ± S.E.M. Number of litters evaluated: 11 C, 14 GHRL and 14 Ant (8 pups/litter; 4 males and four females). *$P < 0.0001$ between all postnatal days. Males: a: $P = 0.028$ GHRL vs C and Ant; females: a = 0.047 GHRL vs C and Ant.
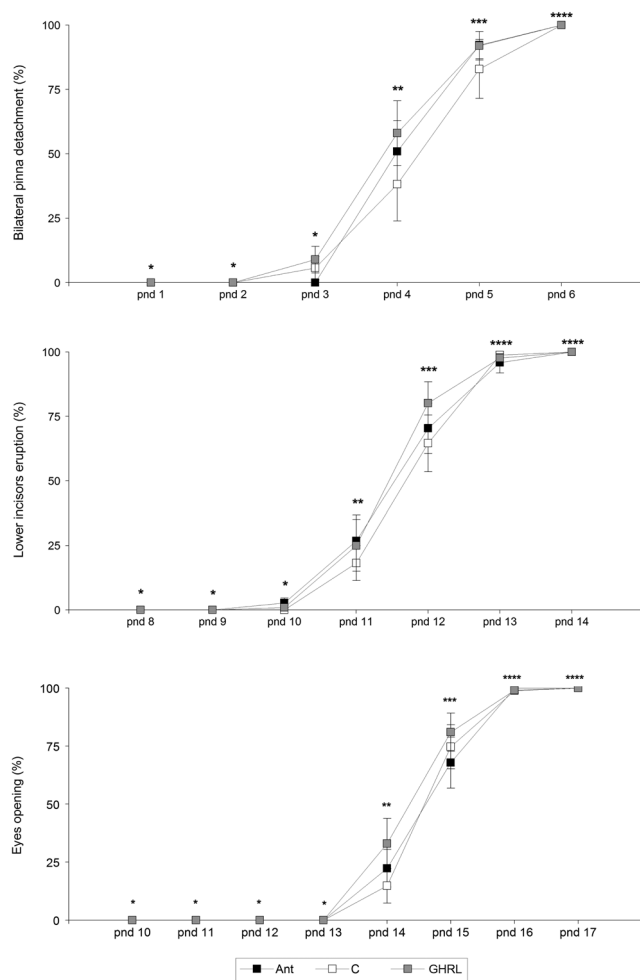
Regarding puberty onset in female pups (i.e. day of vaginal opening), treatments with GHRL and Ant were both characterised by earlier vaginal opening. Nevertheless, the differences reached statistical significance only at pnd 25 (Fig. 3). Ovary volume at pnd 23 was significantly higher in female pups exposed to Ant (Ant = 1085.7 ± 64.0 mm^3 vs GHRL = 663.3 ± 102.8 mm^3 and C = 512.3 ± 116.4 mm^3; $n = 4$–6 animals/treatment; $P = 0.006$).

No differences were detected in the number and length of oestrous cycles (number of oestrous cycles from vaginal opening until adulthood: C = 3.1 ± 0.2, GHRL = 4.0 ± 0.4 and Ant = 3.4 ± 0.3. Duration of oestrous cycles: C = 7.0 ± 0.4, GHRL = 6.4 ± 0.6 and Ant = 6.7 ± 0.4. Length of oestrus: C = 1.7 ± 0.1, GHRL = 1.6 ± 0.1 and Ant = 1.8 ± 0.2; $n = 8$–12 litters/treatment; 2 female pups/litter; $P > 0.05$).

Effects of intragestational GHRL *imbalance on reproductive function of the offspring at adulthood*

At adulthood, male pups from dams treated with Ant exhibited a significantly lower relative testicular weight than those receiving intragestational GHRL. These animals also showed a decrease in sperm motility compared to GHRL or vehicle (Table 3). Nevertheless, these differences did not impact male fertility, which was similar in control pups (percentage of pregnancy: C = 88.8 and Ant = 88.8; litter size: C = 12.0 ± 0.4 and Ant = 12.9 ± 0.6; pups initial weight: C = 0.97 ± 0.03 and Ant = 0.95 ± 0.03; embryo loss: C = 2.1 ± 2.1 and Ant = 3.5 ± 2.3; foetal atrophy: C = 0.5 ± 0.3 and Ant = 0.1 ± 0.1; $P > 0.05$, $n = 9$ males/treatment). No differences were detected either in the testosterone plasma levels of these animals (C = 2.74 ± 1.18 ng/mL, GHRL = 1.35 ± 0.67 ng/mL and Ant = 2.61 ± 0.88 ng/mL; $n = 4$-7 males/treatment, $P > 0.05$).

Reproductive parameters of female pups at adulthood (Table 4) did not show any differences in latency to mate acceptance, ovulation rate or litter size. Although females exposed to intragestational Ant and GHRL exhibited a higher tendency for embryo loss or foetal atrophy, results did not reach statistical significance (Table 4).

Figure 2 Percentage of pups/treatment positive for the physical parameters evaluated. Dams were treated throughout pregnancy with ghrelin (GHRL: 4 nmol/animal/day), an antagonist (Ant: (D-Lys3) GHRP-6; 6 nmol/animal/day) or vehicle (C: isotonic solution). Values are expressed as mean ± s.e.m. of the percentage of pups positive for each parameter. Number of litters evaluated: 11–14/treatment (8 pups/litter; four males and four females). Differences between postnatal days ($P < 0.0001$): * vs ** vs *** vs ****.

Discussion

In this study, we aimed to evaluate the intragestational role of GHRL on the postnatal development of offspring,

groups (pnd 19: Ant = $0.5 \pm 0.0\%$ vs GHRL = $0.4 \pm 0.0\%$ and C = $0.4 \pm 0.0\%$, $n = 5$–10 litters/treatment; $P = 0.038$). It is important to highlight that pups exposed to intragestational GHRL also showed a tendency to earlier testis descent than C-treated pups.

Table 2 Neurobiological parameters of pups from dams treated with intragestational ghrelin or antagonist.

Variable	Control ($n = 12$)	Ghrelin (4 nmol/animal/day) ($n = 16$)	Antagonist (6 nmol/animal/day) ($n = 16$)
Cliff avoidance (%)	98.9 ± 1.0	96.9 ± 2.4	99.2 ± 0.8
Negative geotaxis (%)	96.2 ± 1.9	96.7 ± 2.3	99.2 ± 0.8
Surface righting reflex (%)	100.0 ± 0.0	99.2 ± 0.8	99.2 ± 0.8

Ghrelin or its antagonist ((D-Lys3)GHRP-6) were administered (s.c.) during the whole pregnancy in two injections/day. Control dams were injected with the vehicle in the same regimen (isotonic solution). Pups did not receive further treatment after birth. Neurobiological parameters, cliff avoidance, negative geotaxis and surface righting reflex were evaluated on postnatal days 8, 9 and 10 respectively. Values are expressed as mean ± s.e.m. of the percentage of pups positive for this parameter. In parenthesis: number of litters evaluated (8 pups/litter).

Table 3 Morphometric parameters and sperm functional activity of pups from dams treated with intragestational ghrelin or an antagonist.

Variable	Control (n = 13)	Ghrelin (4 nmol/animal/day) (n = 15)	Antagonist (6 nmol/animal/day) (n = 15)
Body weight (g)	32.95 ± 0.92	33.51 ± 0.72	34.03 ± 0.88
Testicular weight (g)	0.19 ± 0.01	0.21 ± 0.01	0.19 ± 0.01
Relative TW (%)	0.59 ± 0.02	0.63 ± 0.02	0.57 ± 0.01*
Seminal vesicles weight (g)	0.33 ± 0.02	0.34 ± 0.02	0.33 ± 0.02
Relative SVW (%)	1.01 ± 0.05	1.03 ± 0.07	0.99 ± 0.07
Sperm concentration ($\times 10^6$/mL)	16.34 ± 2.16	16.99 ± 1.99	14.74 ± 1.44
Motile spermatozoa (%)	70.85 ± 3.33	75.56 ± 2.98	63.87 ± 3.64[#]
Immature spermatozoa (%)	14.46 ± 2.17	11.33 ± 1.22	14.33 ± 2.52
Viability (%)	84.00 ± 1.33	81.20 ± 2.14	84.87 ± 1.43
PRO (%)	78.77 ± 2.22	81.13 ± 2.79	78.47 ± 2.98
Acrosomal reaction (%)	89.00 ± 3.13	87.20 ± 1.89	90.40 ± 2.07

Ghrelin (GHRL) or its antagonist (Ant: (D-Lys3)GHRP-6) were administered (s.c.) during the whole pregnancy in two injections/day. Control dams were injected with the vehicle in the same regimen (isotonic solution). Pups did not receive further treatment after birth. At adulthood, morphometric (absolute and relative to body weight) and sperm functional parameters were evaluated. Values are expressed as mean ± S.E.M. In parenthesis: number of animals evaluated (two male pups at adulthood/litter).

*$P = 0.041$ vs GHRL; [#]$P = 0.028$ vs C and GHRL.

PRO, hypoosmotic swelling test (% of spermatozoa reactive to hyposmotic solution); acrosome reaction (% of spermatozoa with an intact acrosome); relative SVW, relative seminal vesicles weight; motile spermatozoa (percentage of progressive spermatozoa); immature spermatozoa (% of spermatozoa bended and/or with a cytoplasmic drop); viability (% of alive spermatozoa); relative TW, relative testicular weight.
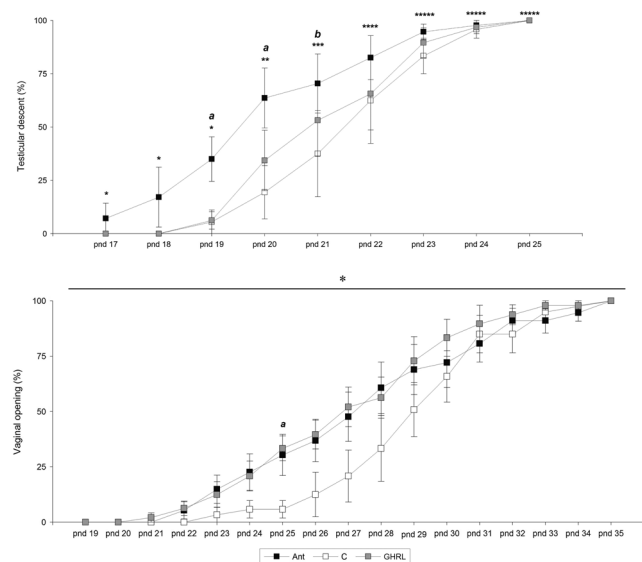

Figure 3 Percentage of pups/treatment positive for the parameter testicular descent or vaginal opening. Dams were treated throughout pregnancy with ghrelin (4 nmol/animal/day), an antagonist ((D-Lys3) GHRP-6; 6 nmol/animal/day) or vehicle (isotonic solution). Values are expressed as mean ± S.E.M. of the percentage of pups positive for each parameter. Number of litters evaluated: 11–14/treatment (8 pups/litter; four males and four females). Testicular descent; differences between treatments: a: $P = 0.046$ Ant vs C and GHRL; b: $P = 0.046$ Ant vs C; differences between postnatal days: $P < 0.0001$: * vs ** vs *** vs **** vs *****. Vaginal opening; differences between treatments: a: $P = 0.033$ Ant and GHRL vs C; differences between postnatal days: *$P < 0.0001$ pnd 19 to 21 vs pnd 23 to 35, pnd 22 vs pnd 24 to 35, pnd 23 vs pnd 25 to 35, pnd 24 vs pnd 26 to 35, pnd 25 and 26 vs pnd 27 to 35, pnd 27 vs pnd 28 to 35, pnd 28 vs pnd 29 to 35, pnd 29 vs pnd 30 to 35, pnd 30 vs pnd 31 to 35, pnd 31 vs pnd 33 to 35, pnd 32 vs pnd 35.

with an emphasis on reproductive programming. For this purpose, we used a mouse model that had been already validated (Bertoldi *et al.* 2011, Luque *et al.* 2014). This model includes a group with supraphysiological concentrations of GHRL (i.e. GHRL), one with endogenous GHRL inhibition (i.e. Ant) and one with the 'normal' concentrations of the peptide (i.e. the C group). Using this approach of GHRL imbalance, we can explore the physiological role of the peptide on foetal programming.

Apart from the increase in pup body weight until adulthood exerted by intragestational exposure to GHRL, the main finding of our study was that GHRL imbalance during pregnancy was able to modify the puberty onset and reproductive function of pups reaching adulthood. These effects were particularly evident in male pups and in the Ant group. Overall, these results support the hypothesis that intragestational GHRL programmes the time of puberty onset and that changes in 'normal' levels (or activity) of GHRL during pregnancy might alter (i.e. accelerate) the timing of this process. This may or may not be interpreted as a 'positive' adaptation, because reproductive function at adulthood was (slightly) poorer in animals exposed to this gestational imbalance.

Our study found that intragestational treatment with Ant (and a similar tendency with GHRL) accelerated sexual development of male pups, evidenced as earlier testis descent and higher testicular weight at peripubertal stages. In female pups, the external signs of puberty onset (i.e. vaginal opening) tended to appear earlier in animals exposed to intragestational Ant or GHRL, but, compared to control animals, varied significantly only at pnd 25. Higher ovary volume was detected in pups born from dams treated with Ant.

There are two arguments that might sustain these results: First, 'intragestational GHRL imbalance modifies

Table 4 Morphometric and reproductive parameters of female pups from dams treated with intragestational ghrelin or an antagonist.

Variable	Control (*n* = 19)	Ghrelin (4 nmol/animal/day) (*n* = 19)	Antagonist (6 nmol/animal/day) (*n* = 22)
Body weight (g)	48.13 ± 1.36	51.93 ± 1.29	52.69 ± 1.91
Days until mate acceptance	2.74 ± 0.25	2.37 ± 0.30	3.37 ± 0.47
Ovulation rate	12.58 ± 0.57	12.74 ± 0.38	12.32 ± 0.42
Viable litter size	12.00 ± 0.52	11.42 ± 0.37	10.86 ± 0.70
Pups weight (g)	0.84 ± 0.03	0.90 ± 0.03	0.81 ± 0.04
Foetal atrophy/female	0.26 ± 0.10	0.42 ± 0.16	0.91 ± 0.46
Females with foetal atrophy/treatment (%)	26.30	31.60	36.40
Embryo loss/female (%)	2.39 ± 1.24	6.06 ± 2.67	4.33 ± 1.63
Females with embryo loss/treatment (%)	21.10	31.60	31.80

Ghrelin or its antagonist ((D-Lys3)GHRP-6) were administered (s.c.) during the whole pregnancy in two injections/day. Control dams were injected with the vehicle in the same regimen (isotonic solution). Pups did not receive further treatment after birth. At adulthood, these females were mated with males without treatment (Day 1 of pregnancy: visible vaginal plug or spermatozoa in vaginal smear). At gestation Day 18, females were killed and reproductive parameters evaluated. Values are expressed as mean ± s.e.m. In parenthesis: number of animals evaluated (2 female pups at adulthood/litter). Body weight: evaluated at pregnancy Day 18; ovulation rate: estimated by the number of corpora lutea at ovaries; foetal atrophy: number of foetuses abnormally smaller than the others; females with foetal atrophy: percentage of females with one or more atrophied foetuses; embryo loss: higher number of corpora lutea than total foetuses (100 − (number foetuses × 100/corpora lutea)); females with embryo loss: percentage of females with embryo loss.

the hypothalamic *Kiss-1* system': it is accepted that kisspeptins are the gatekeeper for puberty onset (or at least, essential amplifiers) and are necessary modulators for GnRH secretion (reviewed in: Chadio & Kotsampasi 2014, De Bond & Smith 2014, Avendaño *et al.* 2017). It has also been recognised that the kisspeptin system links puberty onset/fertility with energy balance (reviewed in: Chadio & Kotsampasi 2014, De Bond & Smith 2014, Avendaño *et al.* 2017). There is considerable evidence that maternal underfeeding, as well as overfeeding, may influence the hypothalamic circuits responsible for reproduction control and the distribution pattern of GnRH neurons, thus affecting puberty and later fertility (Iwasa *et al.* 2010, Castellano *et al.* 2011, Chadio & Kotsampasi 2014, Zambrano *et al.* 2014). Moreover, a link between GHRL and the *Kiss-1* system has been well established. It has been demonstrated, by *in vitro* and *in vivo* models, that GHRL suppresses the amplitude and/or duration of LH peaks by reducing GnRH release, presumably acting by inhibiting the Kiss-1 system (Fernández-Fernández *et al.* 2005, Martini *et al.* 2006). Since it is possible that the *Kiss-1* system might be differentially programmed in our model of GHRL imbalance, we are currently developing new experiments to evaluate the expression of kisspeptin mARN in two hypothalamic nuclei of male/female pups.

Second, 'gonads are a target for intragestational GHRL programming': In rodents, primordial germ cells migrate to the gonadal ridge around gestation Day 12–13. At gestation Day 15.5, these germ cells proliferate and enter meiosis, becoming sex-specific primordial cells (reviewed in Chadio & Kotsampasi 2014). Since the number of female germinal cells is limited by those that enter meiosis, any alteration at this period of foetal development may have long-lasting effects on female progeny (reviewed in Chadio & Kotsampasi 2014). Specifically in males, Sertoli cells

could also provide a target for programming, as they proliferate during the foetal/neonatal and peripubertal periods. During these stages, the mature adult size of the testis and their capacity to produce sperm are established (Orth *et al.* 1988, Chadio & Kotsampasi 2014). In fact, a low number of Sertoli cells and changes in the testicular structure have been observed in adult rats born from dams that underwent undernourishment during gestation and lactation (Genovese *et al.* 2010). Moreover, in prepubertal rats, GHRL has been shown to inhibit immature germ cell proliferation through inhibition of the stem cell factor, which is also a key signal for spermatogenesis (Barreiro *et al.* 2004). Whether such a negative effect of increased GHRL may occur in testicular foetal development remains unknown. The reduced testicular weight at adulthood observed in males exposed to intragestational Ant supports a possible deleterious programming effect at gonadal level. We are currently performing histological analysis of peripubertal and adult offspring's testis in order to elucidate these aspects.

In male pups at adulthood, we also found a reduced sperm motility. This may be explained not only by alterations in spermatogenesis but also by impaired epididymal maturation. This involves changes of the sperm membrane, which provides fertilisation capability and motility (Cooper 1998). Based on basic and clinical studies, we have postulated that the epididymis is a reproductive structure sensitive to nutritional alterations (Martini *et al.* 2007, 2010, Luque *et al.* 2017); whether it is also a target of foetal programming remains unexplored. However, since the differentiation of internal genitalia (including the epididymis) occurs during foetal life, it is a plausible hypothesis (reviewed in Zambrano *et al.* 2014).

Something similar occurs in normal ovary development during embryogenesis, which determines fertility and

reproductive capacity later in life (reviewed in Chadio & Kotsampasi 2014). Female sheep offspring, born from mothers undernourished from mid to late gestation, exhibited fewer corpora lutea. This may potentially affect pregnancy establishment, since the *corpus luteum* provides steroid hormonal support for early pregnancy (Kotsampasi *et al*. 2009, Chadio & Kotsampasi 2014).

Regarding fertility conditions of female pups at adulthood, our study showed a tendency towards increased embryo loss and foetal atrophy in animals exposed to intragestational Ant and/or GHRL. As mentioned before, in an earlier study, we found that the administration of Ant or GHRL (at the same doses used in this study) during early gestation exerted negative effects on preimplantation embryo development and on implantation, evidenced as augmentation of embryo loss and foetal atrophy (Luque *et al*. 2014). It is interesting that a similar tendency was observed in adult females exposed to intragestational GHRL imbalances. A remarkable study by Martin *et al*. (2011) demonstrated a significantly lower implantation index in female mice exposed to intragestational GHRL deficiency (similar to our Ant treatment), explained by impaired endometrial function. As in our study, they did not find any alterations in ovarian follicles or corpora lutea (Martin *et al*. 2011). In a previous study with dams treated with GHRL or Ant from Day 3 to 7 of gestation, we demonstrated that, even though there was a significant increase in embryo loss or foetal atrophy, progesterone levels remained in normal levels (De Loredo *et al*. 2015).

Another important finding of our study is that GHRL imbalance during pregnancy seems to programme body weight increase until adulthood, particularly in male pups. There are some reports that intragestational treatment with GHRL exerts a significant increase in pup body weight at birth. Hayashida *et al*. (2002) and Nakahara *et al*. (2006), treating pregnant rats with GHRL from Day 14–15 to delivery, found a significant increase (of around 10%) in initial pup body weight compared with those treated with vehicle. It is important to remark, nevertheless, that the doses of GHRL used in those studies are much higher than ours (9 nmol/animal/day). In fact, in contrast to our model, these doses increased daily food intake and forced researchers to pair-feed pregnant females to be able to corroborate their results (Nakahara *et al*. 2006). Nevertheless, administering smaller doses of GHRL to rats (1 nmol/animal/day) from Days 1 to 11 of pregnancy, Fernández-Fernández *et al*. (2005) also detected higher initial weight in pups. Additionally, they found a reduction in litter size, which may have modified foetal growth (Fernández-Fernández *et al*. 2005).

In an earlier study using a GHRL analogue (hexarelin 200 μg/animal/day), we observed that hexarelin, administered to mouse dams for the second or third week of pregnancy, increased body weight gain until adulthood without modifying the initial weight (Luque *et al*. 2010). Overall, our results suggest that the GHRL doses used in our study are not high enough to increase foetal growth during pregnancy, but are sufficient to programme body weight gain during postnatal growth. It would be interesting to evaluate pup food intake (which regretfully we did not do in this study), pup metabolism (levels of glucose, insulin, cholesterol, triglycerides, etc.) and/or the hypothalamic orexigenic system (NPY, AgRP, etc.). Variation in these parameters may explain some underlying mechanisms of the differential evolution of body weight.

Finally, the treatment of dams with GHRL or Ant during pregnancy did not modify the physical or neurobiological development of the offspring. Unfortunately, we did not find any other study that analysed these same parameters so that we could compare our results. In a previous study examining the effects of HEX administration throughout pregnancy, we did not find any differences in the parameters evaluated when compared to control subjects (Luque *et al*. 2010).

In summary, our results suggest that intragestational GHRL participates in reproductive foetal programming: alterations in GHRL levels (or activity) during pregnancy hasten puberty onset but exert (or tend to exert) negative effects on adult fertility. This apparent dichotomy of reproductive responses seems to be a common feature of reproductive foetal programming (Chadio & Kotsampasi 2014, De Bond & Smith 2014, Zambrano *et al*. 2014).

To our knowledge, this is the only study that explores the physiological function of GHRL in foetal programming. It should be remembered that many women at reproductive ages are underweight and/or suffer diseases such as anorexia, obesity or polycystic ovarian syndrome. Since all of these pathologies alter GHRL levels (Repaci *et al*. 2011, Goebel-Stengel *et al*. 2013), the study of GHRL as a reproductive programmer is indeed clinically relevant.

Declaration of interest

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