REPRODUCTION
RESEARCH

Reproductive performance of male mice after hypothalamic ghrelin administration

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Abstract

It has been demonstrated that food intake and reproductive physiology are both simultaneously modulated to optimize reproductive success under fluctuating metabolic conditions. Ghrelin (GHRL) is an orexigenic peptide identified as the endogenous ligand of the growth hormone secretagogue receptor that is being investigated for its potential role on reproduction. Considering that data available so far are still limited and characterization of GHRL action mechanism on the reproductive system has not been fully elucidated, we studied the participation of hypothalamus in GHRL effects on sperm functional activity, plasma levels of gonadotropins and histological morphology in mice testes after hypothalamic infusion of 0.3 or 3.0 nmol/day GHRL or artificial cerebrospinal fluid (ACSF) at different treatment periods. We found that GHRL 3.0 nmol/day administration for 42 days significantly reduced sperm concentration (GHRL 3.0 nmol/day = 14.05 ± 2.44 × 10^6/mL vs ACSF = 20.33 ± 1.35 × 10^6/mL, $P < 0.05$) and motility (GHRL 3.0 nmol/day = 59.40 ± 4.20% vs ACSF = 75.80 ± 1.40%, $P < 0.05$). In addition, histological studies showed a significant decrease percentage of spermatogonia (GHRL 3.0 nmol/day = 6.76 ± 0.68% vs ACSF = 9.56 ± 0.41%, $P < 0.05$) and sperm (GHRL 3.0 nmol/day = 24.24 ± 1.92% vs ACSF = 31.20 ± 3.06%, $P < 0.05$). These results were associated with a significant reduction in luteinizing hormone and testosterone plasma levels ($P < 0.05$). As GHRL is an orexigenic peptide, body weight and food intake were measured. Results showed that GHRL increases both parameters; however, the effect did not last beyond the first week of treatment. Results presented in this work confirm that central GHRL administration impairs spermatogenesis and suggest that this effect is mediated by inhibition of hypothalamic–pituitary–gonadal axis.

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Introduction

Ghrelin (GHRL) is a 28 amino acid acylated peptide, mainly produced by stomach (Kojima *et al.* 1999) and in smaller quantities by other tissues including reproductive tissues, such as testicular, ovarian, uterine and placental tissues (Horvath *et al.* 2001, Barreiro *et al.* 2002, Fernandez-Fernandez *et al.* 2004).

It has been recently demonstrated that food intake and reproductive physiology are both simultaneously modulated by multiple chemical signals. In this sense, there are many hormonal mediators and/or neuropeptides that optimize reproductive success under fluctuating metabolic conditions. Gonadal steroids affect energy balance and adiposity in a variety of mammalian species; alterations in nutritional status also markedly influence the hypothalamic–pituitary–gonadal (HPG) axis (Casanueva & Dieguez 1999, Tena-Sempere & Barreiro 2002). Biological actions recognized for GHRL are mostly conducted through interaction with its specific cell-surface receptor, GHS-R1a (Gaytan *et al.* 2004). This receptor is mainly expressed in the arcuate and ventromedial nuclei of the hypothalamus, and it is also present in many peripheral organs (Guan *et al.* 1997, Kojima *et al.* 2001, Gnanapavan *et al.* 2002), indicating multiple GHRL functions on these tissues (Broglio *et al.* 2003). In this line, it has been demonstrated the expression of GHRL receptor in rat testis (Tena-Sempere *et al.* 2002, Barreiro *et al.* 2003). Moreover, *in vitro* studies indicate a direct inhibitory action of GHRL on testicular testosterone secretion (Tena-Sempere *et al.* 2002). In this context, an increasing body of evidence demonstrates that GHRL represents an additional regulatory signal in male reproduction (Garcia *et al.* 2007). Moreover, several lines of evidence suggest that GHRL actions include both systemic effects at different levels of the HPG axis, as well as direct gonadal actions of locally produced GHRL (Barreiro &

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Tena-Sempere 2004, Kheradmand *et al.* 2009). However, data available so far are still limited and characterization of GHRL mechanism of action on the reproductive system remains largely unexplored. Therefore, the present work attempted to recognize hypothalamus participation in GHRL effects on spermatogenesis, reproductive hormones levels (luteinizing (LH), follicle-stimulating (FSH) and testosterone) and sperm quality in mice.

Materials and methods

Animals

Adult male mice (albino Swiss N/NIH), 60 days old with an initial body weight of ≅30 g were used. Colony room was maintained on 14:10-h light–darkness photoperiod and under controlled temperature (24 ± 2°C), with pelleted mouse food (Gepsa Feeds, Pilar, Argentina) and water *ad libitum*.

Experiments were conducted in accordance with the guidelines of the experimental protocol approved by the Committee for the Care and Use of Experimental Animals, School of Medical Sciences, National University of Córdoba (07/07/15).

Surgery

After 2 weeks of adaptation period in the storage room, ventromedial hypothalamic surgery was carried out using osmotic pumps (Alzet, Durect, Cupertino, CA, USA), according to the methods described by Paxinos and Franklin (2001). Animals were anesthetized using a combination of 55 mg/kg ketamine HCl (Vetanarcol König: Laboratories König S.A, Argentina) and 11 mg/kg xylazine (Kensol König: Laboratories König S.A) and placed in a stereotaxic apparatus. The coordinates relative to bregma were anterior 0.15 mm, lateral 0.05 mm and vertical 5.5 mm. Cannulae were fixed to the skull surface with dental acrylic cement. Different animals were infused daily with ACSF or GHRL 0.3 or 3.0 nmol/day. After surgery, animals were housed in individual cages.

At the end of the experiments, all brains were subjected to histological analysis to confirm the guide cannula location (Fig. 1). Only animals with correct cannula position were considered for statistical analysis.

Drugs and treatment

GHRL (acyl-ghrelin) (Innovagen, Sweden) was dissolved in ACSF (0.3 or 3.0 nmol/μL), divided into aliquots and kept at −20°C until experimental day. GHRL was infused chronically in the hypothalamus employing osmotic pumps model 1007D (0.5 μL/h, 7 days) or model 2006 (0.15 μL/h, 42 days).

Experimental procedure

In mouse, spermatogenesis consists in a developmental period of 35 days from differentiated type A1 spermatogonia to spermatozoa (Franca *et al.* 1998), while each spermatogenic cycle length is 8.6 days (Clermont & Trott 1969). Taking this

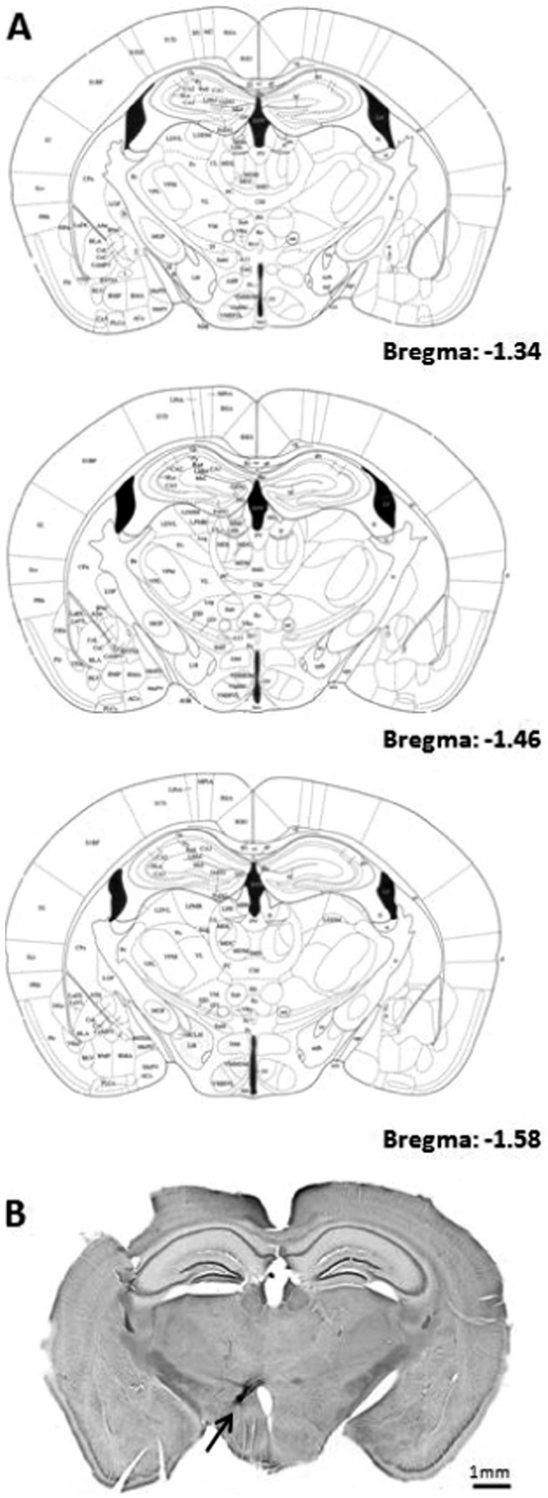

Figure 1 Histological confirmation of cannula location into the hypothalamus. (A) Coronal sections of mice brain based on the atlas of Paxinos and Franklin (2001). Anteroposterior locations relative to the bregma are indicated in each section. (B) Infusion position (arrow) employing osmotic pumps model 2006 (0.15 μL per hour, 42 days). Photography obtained by spectral confocal microscope (FV1200 OLYMPUS), Ob: 4× with PRIOR motorized stage assembled to software ASW 4.0. Scale bar: 1 mm.

into account, assessment of GHRL effects on sperm functional activity in mice was carried out using different experimental approaches. Specifically for this purpose, two sets of experiments were performed. They are the following.

First, in order to study hypothalamus participation in GHRL effects on epididymal maturation, animals were treated for 7 days (period that covers the release of mature spermatozoa into the lumen of the seminiferous tubule and a series of cellular modifications that occur during transit along the epididymal tubule).

Second, in order to study hypothalamus participation in GHRL effects on spermatogenesis, animals were treated for 42 days (period that covers at least one complete spermatogenic cycle and epididymal sperm migration).

In order to minimize potential bias of major differences in body weight on the different reproductive endpoints under analysis, food intake and body weight were daily measured in both experimental sets. After treatment, animals were killed by decapitation in a room separate from that in which the other animals were kept. Trunk blood was collected into EDTA tubes and immediately centrifuged (1700 rpm, 10 min, 4°C). Individual plasma samples were frozen and stored (at −20°C) for subsequent hormonal determination.

Spermatozoa were obtained by making incisions in the isolated caudal portion of the epididymis, allowing the sperm to extrude into 2 mL of Tyrode medium for 10 min.

Testes were immediately removed and weighed (free of surrounding fat). Weight is expressed as relative weight (testicular weight/animal weight). Histological and ultrastructural studies were then performed in animals treated for 42 days.

Food intake and body weight

Food intake was determined as the difference between food available and food remainder 24 h later between 11:00 and 12:00 h to prevent variations determined by circadian rhythms. Body weight was measured daily. Data are expressed in grams (g) as mean ± S.E.M. $n = 9–12$ animals/group.

Incubation medium

Modified Tyrode's medium (Fraser 1993) supplemented with 4 mg/mL of bovine serum albumin (Sigma, St Louis, MO, USA) was employed and gametes were incubated at 37°C (5% CO_2:95% air) and 100% humidity until use.

Reproductive parameters

Sperm concentration, motility and maturity were measured in a Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel) under an inverted microscope (Olympus CK2) at 200× magnification (Makler 1980). Concentration is expressed in millions of spermatozoa per mL of sperm suspension. Motility results are expressed as a percentage of motile cells (progressive plus non-progressive spermatozoa). No less than 100 gametes were examined (Fiol de Cuneo *et al.* 1994). To evaluate sperm maturity, percentages of bending spermatozoa (with a marked flagellar angularity of 90–180° toward the tail in the sperm head or midpiece), spermatozoa with cytoplasmic droplets (a remnant of germ cell cytoplasm in the sperm tail), and those with both characteristics were quantified. $n = 9–10$ animals/group.

Sperm viability

Sperm viability was evaluated by supravital staining with Hoechst 33258 (H258) (3 mg/mL in isotonic solution) (Calbiochem) (Yelian & Dukelow 1992). Using appropriate ultraviolet fluorescence optics (Axiolab, Zeiss, Germany), spermatozoa showing bright fluorescent nuclei were scored as dead and cells that excluded the H258 were scored as viable. Viability of at least 100 cells was assessed; results are expressed as a percentage of viable spermatozoa. $n = 9–10$ animals/group.

Hypoosmotic swelling test

As previously described (Ruiz *et al.* 1996), 0.1 mL of sperm suspension was mixed with 1 mL of the hypoosmotic solution (100 mosmol/L) for 45 min (37°C). Evaluations were made by phase-contrast microscopy at a magnification of 400×; 100 or more cells were observed; results are expressed as the percentage of spermatozoa that showed tail swelling ($n = 9–10$ animals/group).

Acrosomal integrity

Samples were treated as stated by Puechagut *et al.* (2012). Acrosomal integrity was evaluated with double staining with *Pisum sativum* agglutinin labeled with fluorescein isothiocyanate (FITC-PSA) (Sigma) and Hoechst 33258. Only viable cells were scored; results are expressed as the percentage of acrosome-intact cells ($n = 9–10$ animals/group).

Hormone assays

Gonadotropins assay

Luteinizing hormone and stimulating follicle hormone concentrations in plasma samples were determined using a commercial ELISA kit following the manufacturer's instructions (MyBioSource, USA). The range of detection for the LH kit was 0.78–50 mIU/mL and for FSH kit 3.12–100 mIU/mL. The minimum detectable Mouse LH up to 0.08 mIU/mL and the lowest detection limit for FSH was 1.0 mIU/mL. Intra- and interassay coefficients of variation for LH were ≤8% and ≤12% respectively. Both intra- and interassay coefficients of variation for FSH were <15%. Results were expressed in mIU/ml. $n = 5–7$ animals/group.

Testosterone assay

Testosterone concentrations in plasma samples were determined by enzyme immunoassay (EIA) test, using polyclonal antibodies, standards and their corresponding horseradish peroxidase conjugates (anti-Testosterone R156/7, Department of Population Health and Reproduction, C. Munro, UC Davis, CA, USA). Antibody cross-reacts with 5-α-dihydrotestosterone (57.4%), androstenedione (0.27%), androsterone (0.04 %), cholesterol (0.03%) and <0.02% with all other steroids tested. Assays were performed according to

general technique described by Munro and Lasley (1988). Briefly, plasma samples were assayed in duplicate using flat-bottom microtiter plates (Nunc Maxisorp, VWR, Mississauga, ON, Canada). Plates were first coated with 50 µL of the antibody stock 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, Winooski, VT, USA). Immediately after washing, 50 µL of 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 (21°C) 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-ethylbenzthiazoline sulfonic acid) diammonium salt, pH 4.0) were added to each well (Munro *et al*. 1991). Absorbance was measured at 405 nm using a microplate reader (Thermo Electron Corporation, Thermo Fisher Scientific, Waltham, MA, USA). Accepted intra- and interassay coefficients of variation for the high and low control samples were <10 and <15%, respectively, in all hormonal determinations. Assay sensitivity was 2.4 pg/well. Results were expressed as Log10 of the plasma testosterone concentration in ng/mL ($n = 9$–10 animals/group).

Testicular histology

Testes were fixed in Bouin's solution for 48 h, dehydrated and embedded in paraffin. Histological sections (5 mm) were stained with hematoxylin and eosin (H&E). All sections were observed with optical Zeiss Primo Star trinocular microscope (Gottingen, Germany) connected to Zeiss Axio Cam ERc 5s digital camera. The H&E-stained sections were used to identify different stages of the seminiferous epithelium cycle, Leydig and Sertoli cells. Differential cell counts were gathered from every 20th section to provide a 5% sample selection per testis. Histomorphometry was conducted by counting of 20 seminiferous tubules in each slide (Kheradmand *et al*. 2009). Results are expressed as percentage of each germ cell type per animal and Leydig and Sertoli cells as number of cell per animal ($n = 5$ animals/group).

Testicular morphometry analyses were performed taking into account 50 round or nearly round seminiferous tubules from each mouse (Duan *et al*. 2014). Seminiferous tubular diameter and epithelium height were measured with AxioVision 4.0 V 4.8.2.0 image analysis system. Results are expressed in micrometers ($n = 5$ animals/group).

Transmission electron microscopy

As stated by Diaz de Barboza (2014), testes were fixed by immersion in a mixture of 2% (v/v) glutaraldehyde and 4% (v/v) formaldehyde in 0.1 M cacodylate buffer, treated with 1% w/v osmium tetroxide for 2 h, dehydrated and embedded in Araldite resin at 60°C for 48 h. For ultrastructural studies, thin sections were cut with a diamond knife on a JEOL JUM-7 ultramicrotome and examined in a Zeiss LEO-906E electron microscope (Oberkochen, Germany) ($n = 3$–5 animals/group).

Statistical analysis

Data were analyzed using STATISTICA – Stat Soft (version 10) statistical package. Normality and homogeneity of variances were corroborated; when statistical assumptions were violated, data were transformed to Log10. Data are expressed as mean ± S.E.M.; P values lower than 0.05 were considered statistically significant.

Reproductive parameters and hormone assays data were analyzed by one-way ANOVA and LSD test for *post hoc* comparison was performed when appropriate. When data were expressed as percentages, they were analyzed using chi-square test.

Food intake and body weight data were analyzed using a repeated-measure ANOVA and LSD *post hoc* test was performed when appropriate.

Differential cell counts from histological data were analyzed by multi-way ANOVA and LSD *post hoc* test was performed when appropriate.

Results

Food intake and body weight

Effects of 7-day hypothalamic GHRL administration on mice food intake and body weight

Quantification of food intake carried out during the experimental period is shown in Fig. 2A. As can be seen, GHRL 3.0 nmol/day treatment induced significant differences in the amount of daily food consumed only between the second and the fifth day of treatment ($P < 0.05$). Repeated-measures ANOVA also revealed a significant interaction between treatment and time ($F = 2.60$, $df = 12$, $P < 0.05$), significant effects of treatment ($F = 6.73$, $df = 2$, $P < 0.05$) and significant effects of time ($F = 51.70$, $df = 6$, $P < 0.05$).

In relation to body weight (Fig. 2B), the higher intrahypothalamic GHRL dose employed for 7 days induced significant change on body weight at the end of treatment (ACSF: 33.16 ± 0.81 g, $n = 11$ vs GHRL 3.0: 39.95 ± 1.17 g, $n = 9$ animals/group; $F = 7.89$, $df = 2$, $P < 0.05$).

Effects of 42-day hypothalamic GHRL administration on mice food intake and body weight

Quantification of food intake carried out daily during the experimental period is shown as weekly food intake in Fig. 3A. Repeated-measures ANOVA test revealed a significant interaction between GHRL treatment and time ($F = 3.72$, $df = 10$, $P < 0.05$), significant effects of time ($F = 10.65$, $df = 5$, $P < 0.05$) and no significant effects of treatment ($P > 0.05$). As can be seen, GHRL 3.0 nmol/day treatment induced significant increase in the amount of food consumed only during the first week of treatment ($P < 0.05$).

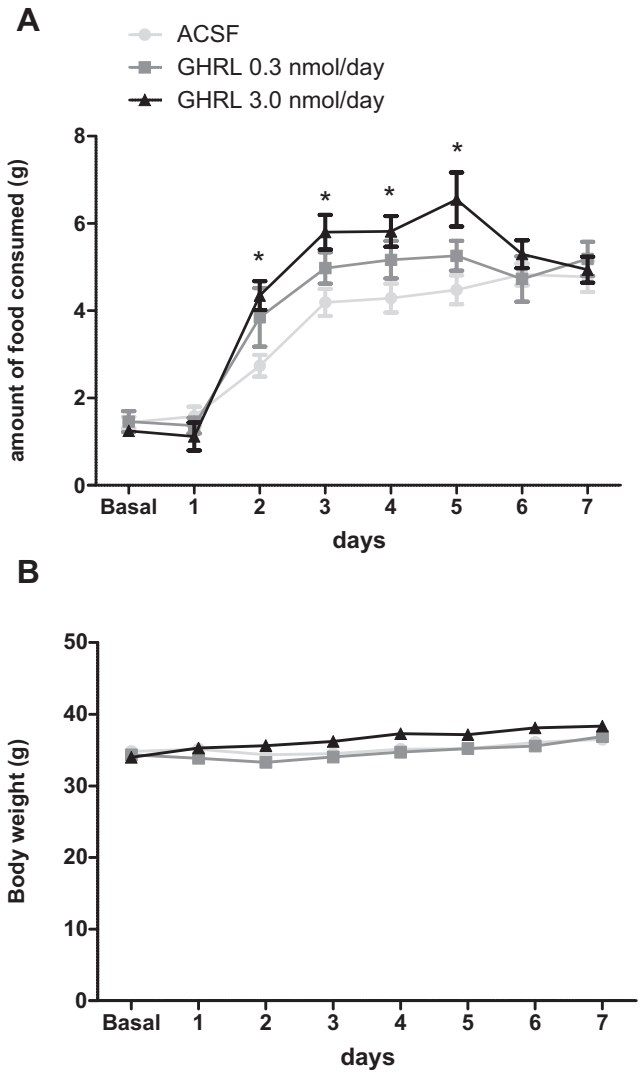

Figure 2 Ghrelin (GHRL) effect on daily food intake (A) and body weight (B) in mice chronically treated for 7 days (mean (g) ± S.E.M.). Animals were infused into hypothalamus with GHRL or artificial cerebrospinal fluid (ACSF) employing osmotic pumps model 1007D (0.5 µL per hour, 7 days). $n = 9–12$ animals/group. *Significant differences in comparison to ACSF animals, $P < 0.05$.

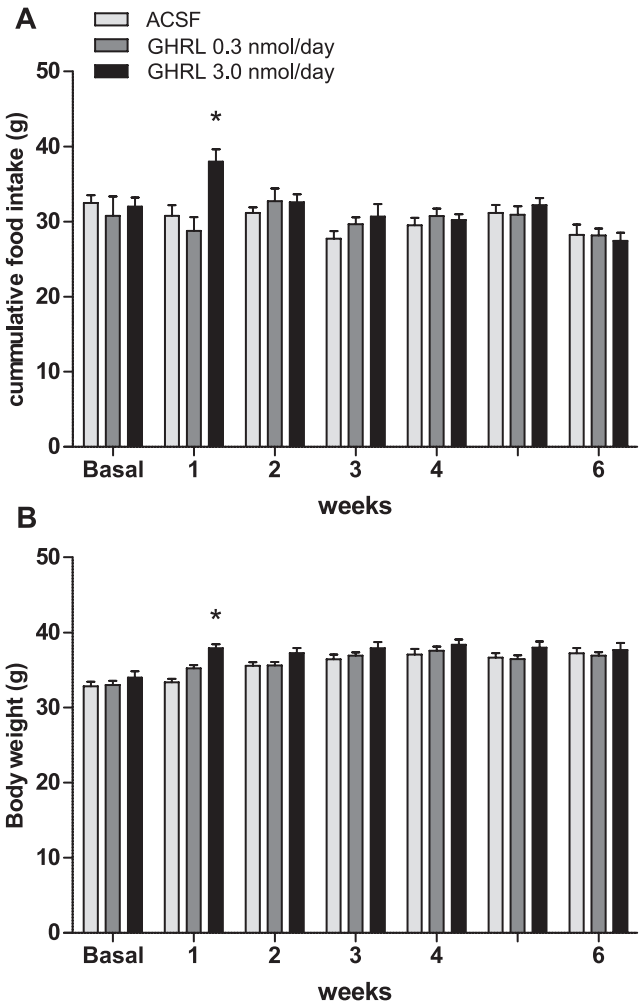

Figure 3 Ghrelin (GHRL) effect on weekly cumulative food intake (A) and body weight (B) in mice chronically treated for 42 days (mean (g) ± S.E.M.). Animals were infused into hypothalamus with GHRL or artificial cerebrospinal fluid (ACSF) employing osmotic pumps model 2006 (0.15 µL/h, 42 days). $n = 9–10$ animals/group. *Significant differences in comparison to ACSF animals, $P < 0.05$.

Concordantly with increased food intake, a significant increase in body weight in GHRL 3.0 nmol/day vs ACSF group was detected only in the first week of treatment (Fig. 3B) ($F = 2.98$, $df = 2$, $P < 0.05$). However, no significant variations were found between final and initial weight for each animal ($P > 0.05$).

Reproductive parameters

Effects of 7-day hypothalamic GHRL administration on mice sperm functional activity

Evaluation of seminal parameters showed no significant modification in the parameters evaluated between groups (sperm concentration, motility, viability, response to hypoosmotic shock and acrosomal integrity, $P > 0.05$). Moreover, there were no significant differences in sperm maturity parameters like percentages of bending spermatozoa, spermatozoa with cytoplasmic droplets or those with both characteristics (Table 1).

Relative testicular weights were similar in all the three groups (4.97 ± 0.20; 5.79 ± 0.12, 5.54 ± 0.16 mg in ACSF, GHRL 0.3 and GHRL 3.0 nmol/day animals respectively).

Effects of 42-day hypothalamic GHRL administration on mice sperm functional activity

Figure 4 shows the effects of hypothalamic GHRL treatment for 42 days on sperm concentration (upper panel) and percentage of sperm motility (lower panel), respectively. As can be seen, GHRL 3.0 nmol/day significantly reduces sperm concentration ($F = 3.93$,

Table 1 Functional activity of caudal epididymis sperm from mice treated for 7 days with artificial cerebrospinal fluid or Ghrelin.

	ACSF	**GHRL** (0.3 nmol/day)	**GHRL** (3.0 nmol/day)
Sperm concentration (1×10^6/mL)	23.59 ± 1.90	18.53 ± 2.06	25.11 ± 6.11
Motility (%)	75.00 ± 5.25	81.80 ± 2.24	76.94 ± 2.45
Viability (%)	75.89 ± 5.78	77.50 ± 5.09	87.89 ± 1.84
Hypoosmotic swelling test (%)	74.72 ± 3.62	80.82 ± 1.60	79.56 ± 1.94
Acrosomal integrity (%)	67.89 ± 6.77	67.40 ± 6.70	78.67 ± 6.28
Bending (%)	2.67 ± 0.85	4.40 ± 1.63	3.28 ± 0.68
Cytoplasmic droplets (%)	11.17 ± 2.50	13.10 ± 3.52	15.00 ± 5.96

Animals were infused with Ghrelin (GHRL) or artificial cerebrospinal fluid (ACSF) into hypothalamus employing osmotic pumps model 1007D (0.5 µL/h, 7 days). Results are expressed as mean ± S.E.M. $n = 9$–10 animals/group.

$df = 2$, $P < 0.05$) and motility ($F = 12.82$, $df = 2$, $P < 0.05$). There were no significant differences between groups in the other parameters evaluated (viability, response to hypoosmotic shock and acrosomal integrity) (Table 2).

GHRL effect on plasma levels of gonadotropins in mice chronically treated for 7 or 42 days

Figure 5 shows GHRL effect on FSH plasma levels in animals treated for 7 (Fig. 5A) or 42 (Fig. 5B) days. Similar FSH plasma levels were observed in animals treated with GHRL in relation to ACSF administered mice ($P > 0.05$).

Figure 6 shows GHRL effect on plasma LH levels in animals treated for 7 (Fig. 6A) or 42 (Fig. 6B) days. As can be seen, 7-day treatment produced no significant changes in plasma LH levels ($P > 0.05$) while GHRL 3.0 nmol/day treatment for 42 days significantly reduced this variable ($F = 4.21$, $df = 2$, $P < 0.05$).

GHRL effect on plasma testosterone levels in mice chronically treated for 7 or 42 days

Figure 7 shows GHRL effect on plasma testosterone levels in animals treated for 7 (Fig. 7A) or 42 (Fig. 7B) days. As can be seen, 7-day treatment produced no significant changes in testosterone plasma levels ($P > 0.05$) while GHRL 3.0 nmol/day treatment for 42 days significantly reduced this variable ($F = 4.98$, $df = 2$, $P < 0.05$).

In line with results previously presented, linear correlation analysis showed significant correlation between epididymal spermatozoa concentration (10^6 cells/mL) and the log10 of plasma testosterone concentration (ng/mL) ($r = 0.8386$) and between sperm motility (%) and the log10 of plasma testosterone concentration (ng/mL) ($r = 0.8031$) (Fig. 8).

Testicular histology and ultrastructural morphology

Effects of 42-day hypothalamic GHRL administration on mice testicular histology

Figure 9 shows GHRL effect on cell spermatic cycle. As can be seen, only GHRL 3.0 nmol/day treatment induced significant decrease in the percentage of spermatogonia and sperm ($F = 3841.96$, $df = 4$, $P < 0.05$) and an increase in the intermediate stages, spermatocytes and spermatids ($P < 0.05$). There were no significant differences between GHRL doses ($P > 0.05$). No significant differences were

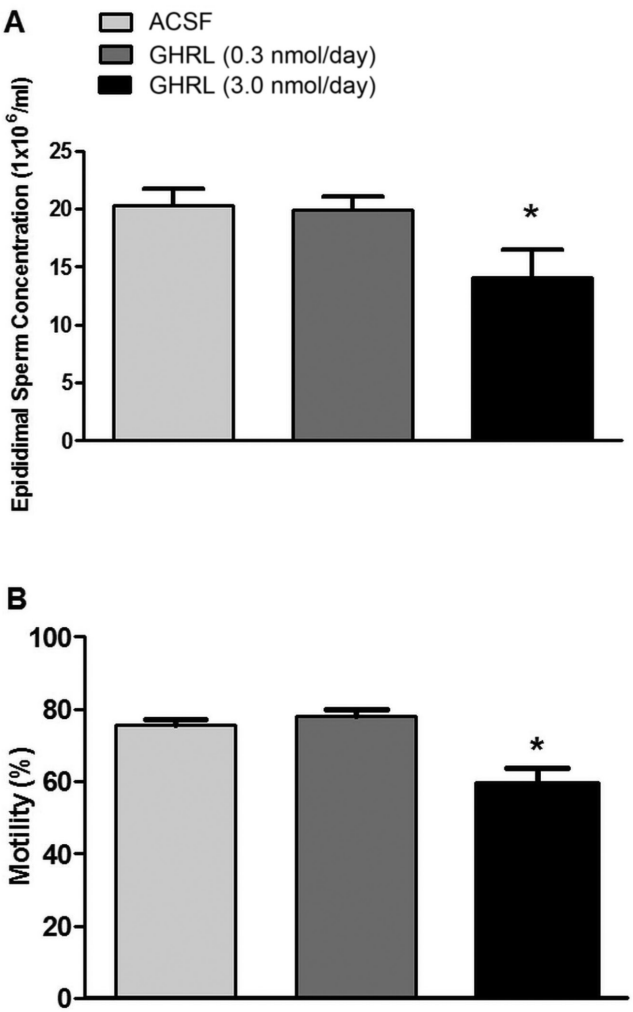

Figure 4 Ghrelin (GHRL) effect on sperm concentration (1×10^6/mL) (A) and percentage of sperm motility (B) in mice chronically treated for 42 days. Animals were infused into hypothalamus with GHRL or artificial cerebrospinal fluid (ACSF) employing osmotic pumps model 2006 (0.15 µL/h, 42 days). Results are expressed as mean ± S.E.M. $n = 9$–10 animals/group. *Significant differences in comparison to ACSF animals, $P < 0.05$.

Table 2 Functional activity of caudal epididymis sperm from mice treated for 42 days with artificial cerebrospinal fluid or Ghrelin.

	ACSF	**GHRL** (0.3 nmol/day)	**GHRL** (3.0 nmol/day)
Viability (%)	83.40 ± 2.09	79.55 ± 2.33	78.30 ± 2.00
Hypoosmotic swelling test (%)	71.91 ± 2.42	72.91 ± 2.81	69.05 ± 3.98
Acrosomal integrity (%)	71.00 ± 6.23	71.78 ± 4.36	68.40 ± 7.37

Animals were infused into hypothalamus with Ghrelin (GHRL) or artificial cerebrospinal fluid (ACSF) employing osmotic pumps model 2006 (0.15 µL/h, 42 days). Results are expressed as means ± S.E.M. of the percentage. $n = 9–10$ animals/group.

found in the number of Sertoli cells or Leydig cells ($P > 0.05$) (Table 3).

The ANOVA revealed no significant differences on morphometric parameters in mice treated with GHRL when compared with control animals ($P > 0.05$) (Table 4).

No significant differences were found in relative testicle weights (4.97 ± 0.18, 5.15 ± 0.13, 4.99 ± 0.17 mg in ACSF, GHRL 0.3 and GHRL 3.0 nmol/day animals, respectively).

Effects of 42-day hypothalamic GHRL administration on mice testicular ultrastructure

As can be seen in Fig. 10, animals treated with GHRL 3.0 nmol/day showed changes in spermatozoa nucleus where chromatin presented an abnormal condensation pattern. In addition, the morphology of the acrosome was altered. Also, a large amount of elongated spermatids can be observed, which is evidenced by the presence of a transient structure called the manchette (microtubules that extend parallel to the major axis of the cell around the posterior part of the nucleus and the upper part of the flagellum).

In addition, no morphological differences were observed in Leydig cells in both control and treated groups.

Discussion

Studies performed to date regarding the GHRL role in reproductive physiology have been mostly restricted to direct gonadal actions of locally produced GHRL or peptide effects on reproductive hormones, while central effects on functional sperm activity have not been fully elucidated so far. The present work studies the effect of chronic GHRL administration on reproductive hormones concentration, spermatogenesis and sperm quality in mice. We found that hypothalamic GHRL 3.0 nmol/day administration for 42 days decreased caudal epididymal sperm concentration and motility, without affecting sperm viability and parameters related with sperm membrane functionality, such as response to hypoosmotic swelling test and acrosomal integrity. Results also show that GHRL significantly reduced LH and testosterone plasma levels and, moreover, decreased the percentage of spermatogonia and sperm but increased the intermediate forms, spermatocytes and spermatids after 42 days of treatment. These results provide evidence that chronic central GHRL administration impairs spermatogenesis and that this effect is potentially mediated by inhibition of the HPG axis, since we observed a reduction on plasmatic levels of LH. Function of the HPG axis, and hence of the gonads, depends on strict hormonal control to generate fertilizable gametes and ensure male fertility (Saez 1994, Abou Heif *et al.* 2010). Increasing evidence suggests that GHRL may participate in such regulatory

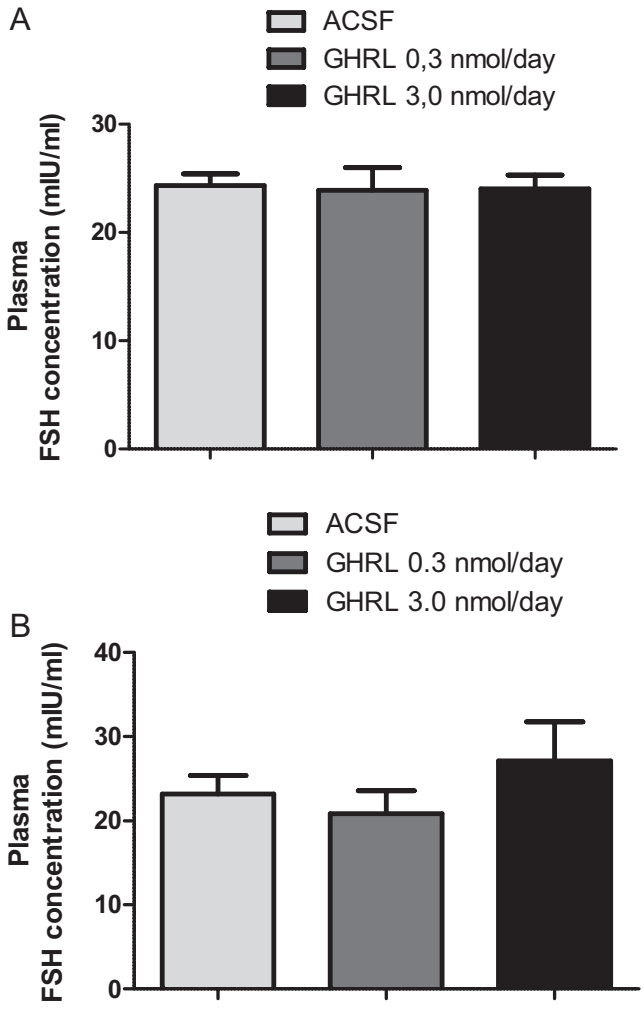

Figure 5 Ghrelin (GHRL) effect on plasma FSH concentration (mIU/mL) in mice chronically treated for 7 (A) or 42 (B) days. Animals were infused into hypothalamus with GHRL or artificial cerebrospinal fluid (ACSF) employing osmotic pumps model 2006 (0.15 µL/h, 42 days). Results are expressed as mean ± S.E.M. $n = 5–7$ animals/group. *Significant differences vs ACSF animals, $P < 0.05$.

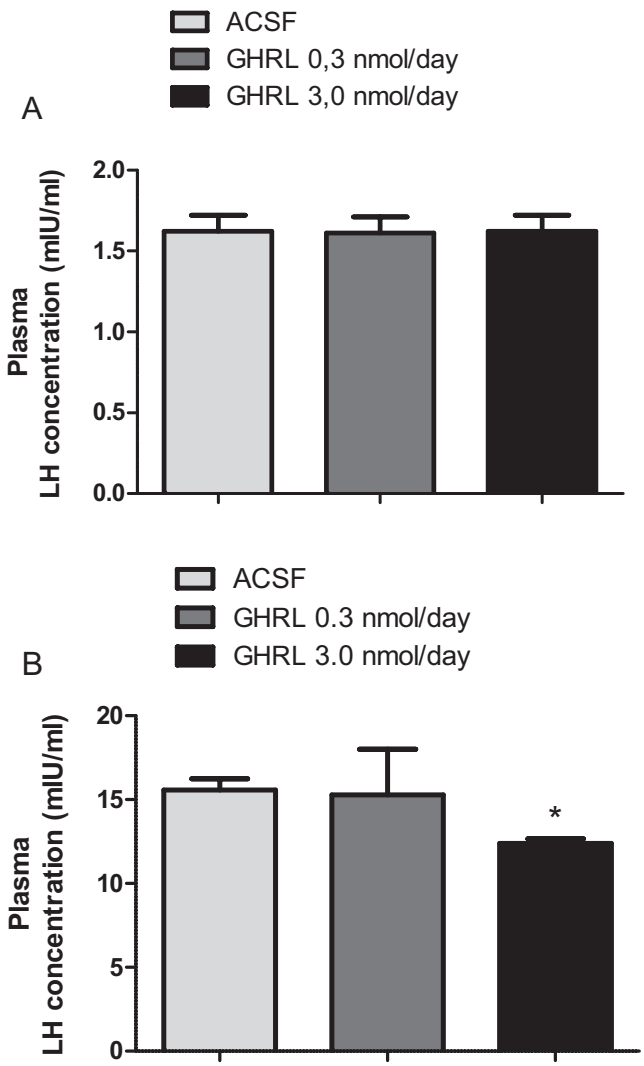

Figure 6 Ghrelin (GHRL) effect on plasma LH concentration (mIU/mL) in mice chronically treated for 7 (A) or 42 (B) days. Animals were infused into hypothalamus with GHRL or artificial cerebrospinal fluid (ACSF) employing osmotic pumps model 2006 (0.15 µL per hour, 42 days). Results are expressed as mean ± S.E.M. $n = 5–7$ animals/group. *Significant differences vs ACSF animals, $P < 0.05$.

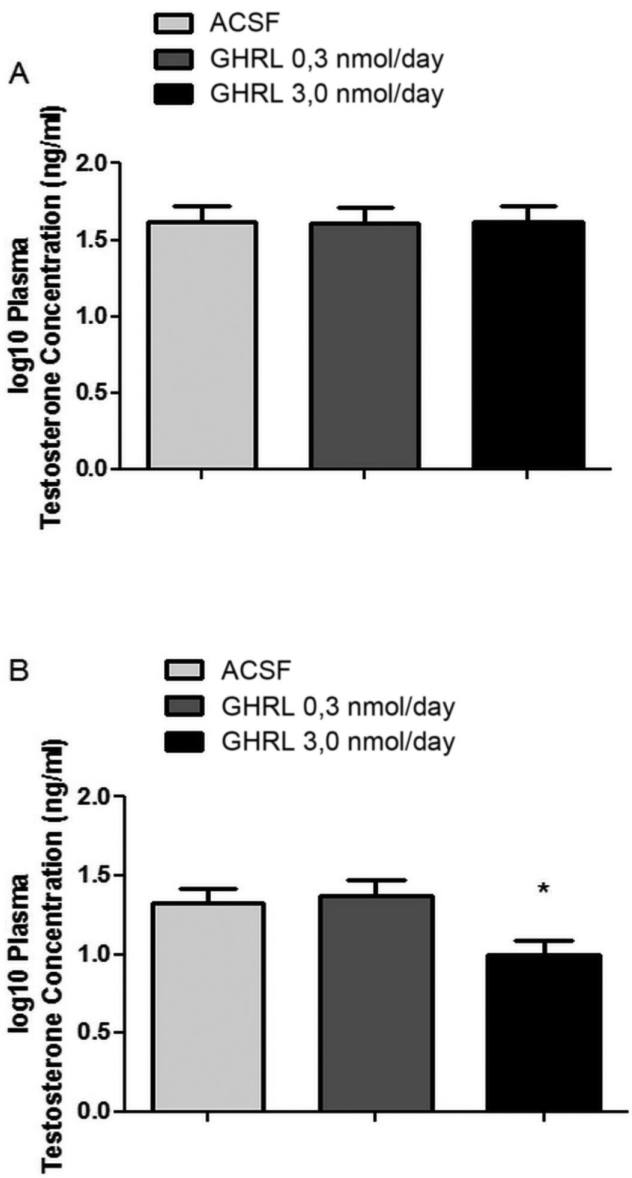

Figure 7 Ghrelin (GHRL) effect on plasma testosterone concentration in mice chronically treated for 7 (A) or 42 (B) days. Animals were infused into hypothalamus with GHRL or artificial cerebrospinal fluid (ACSF) employing osmotic pumps model 2006 (0.15 µL/h, 42 days). Results are expressed as log10 of the plasma testosterone concentration in ng/mL. $n = 9–10$ animals/group. *Significant differences vs ACSF animals, $P < 0.05$.

network (Tena-Sempere *et al.* 2002, Tena-Sempere 2005, 2007). Thus, central GHRL could be regulating the cellular remodeling that occurs as spermatozoa migrate across the epididymis, acting through hormonal control of the HPG axis. Modifications during epididymal transit result in the acquisition of motility and the ability to become capacitated for fertilization (Sullivan *et al.* 2007). Decreased motility observed in sperm of animals treated with Grh 3.0 nmol/day suggests that GHRL effect could be related to changes in testosterone levels induced by the peptide. These results are supported by high correlation between the concentration of epididymal spermatozoa and testosterone concentration in plasma.

Production of fertile spermatozoa is the result of numerous stages of cell differentiation. In the testis, seminiferous tubules and interstitial tissues cooperate in spermatogenic process. Leydig cells are the major interstitial cells and synthesize testosterone for spermatogenesis (Ozawa *et al.* 2002). It has been demonstrated that intratesticular GHRL injection *in vivo* is able to inhibit proliferative rate of immature Leydig cells (Barreiro *et al.* 2004). In this line, subcutaneous injection of GHRL (1 nmol/day for 10 days) in adult

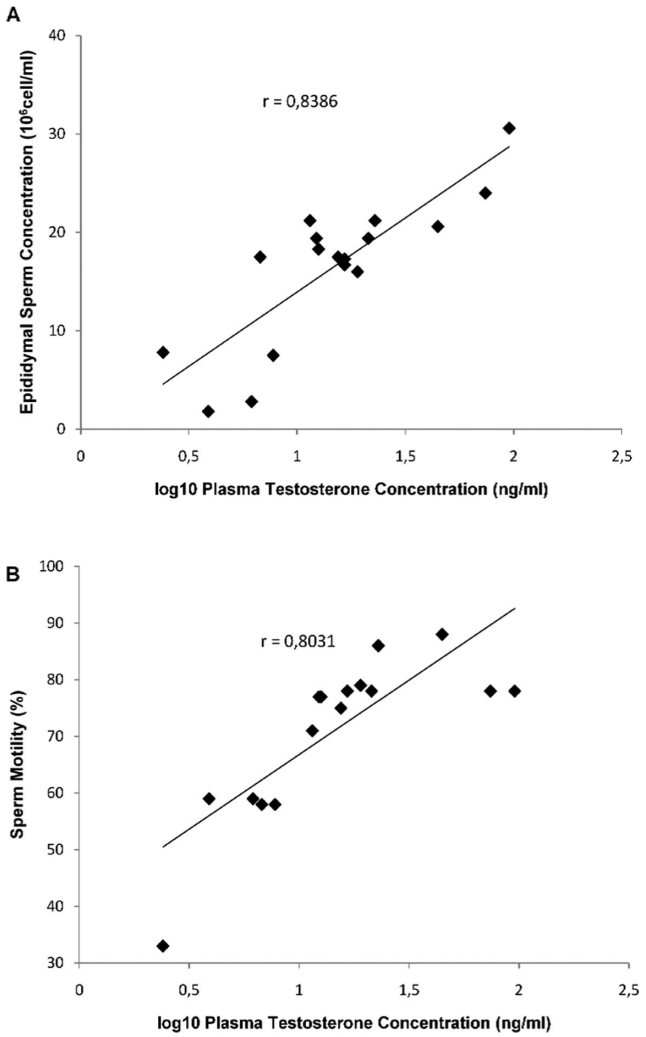

Figure 8 Correlation between log10 plasma testosterone concentration (ng/mL) and epididymal sperm concentration (A, spearman rank correlation coefficient $r = 0.8386$; $P < 0.05$) or sperm motility (B, $r = 0.8031$; $P < 0.05$) in mice chronically treated for 42 days.

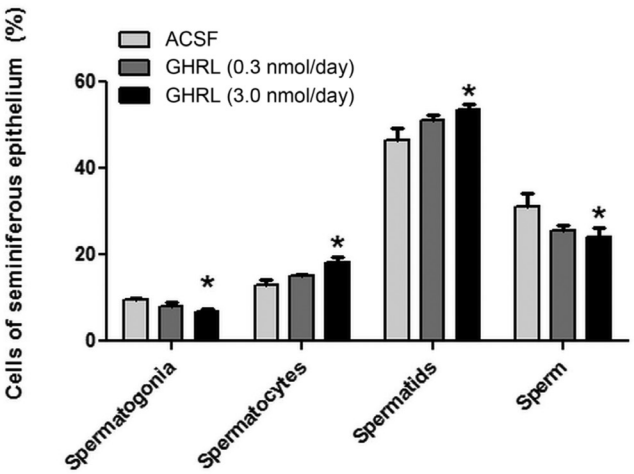

Figure 9 Ghrelin (GHRL) effect on percentage of germ cell types present in the seminiferous epithelium in mice chronically treated for 42 days. Animals were infused with GHRL or artificial cerebrospinal fluid (ACSF) employing osmotic pumps model 2006 (0.15 µL/h, 42 days). Differential cell counts were gathered counting of at least 20 seminiferous tubules in each slide. Results are expressed in percentage of each cell type as mean ± S.E.M. $n = 5$ animals/group. *Significant differences in comparison to ACSF animals, $P < 0.05$.

rats showed a decreased number of different spermatic cells as well as Sertoli or Leydig cells and numerous intracellular changes that confirm the suppression of the functional capacity of these cells (Kheradmand *et al.* 2009). In contrast with this, our results did not show changes either in the number of Sertoli or in Leydig cells, supporting the hypothesis that the changes observed in our experimental model could be a consequence of reproductive hormone regulation by GHRL. Differences in experimental models, administration protocols (acute, sub-chronic or chronic), doses or administration time could be responsible for some inconsistencies in the reports.

It is well known that both FSH and LH are necessary for the initiation of spermatogenesis and the establishment of fertility in men (Matthiesson *et al.* 2006). It has been reported that spermatogonial maturation is largely FSH dependent (Weinbauer *et al.* 1991, Haywood *et al.* 2003, Meachem *et al.* 2005); spermiogenesis appears to be reliant on LH/intratesticular testosterone (O'Donnell *et al.* 1994, 1996), whereas spermiation, the process by which mature sperm are released from the Sertoli cell into the lumen of the seminiferous tubule, requires both hormones (Saito *et al.* 2000). Regarding this, loss of both FSH and LH action in the GnRH-deficient mouse (Singh *et al.* 1995) or LH action alone (Lei *et al.* 2001, Zhang *et al.* 2001, Ma *et al.* 2004) results in germ cell arrest at spermatocyte or spermatid stages. Thus, germ cell development in the mouse can be completed in the absence of FSH but not androgen action (Matthiesson *et al.* 2006). Such data support results observed in our experiments about spermatid retention in relation to the overall reduction of sperm production caused by low plasmatic testosterone concentration dependent on LH stimulation. Therefore, current findings are similar to results previously discussed, and GHRL effects on reproductive axis seem

Table 3 Ghrelin effect on number of Leydig and Sertoli cells in mice chronically treated for 42 days.

	ACSF	GHRL (0.3 nmol/day)	GHRL (3.0 nmol/day)
Leydig cells	65.60 ± 7.15	78.40 ± 6.33	70.20 ± 7.77
Sertoli cells	11.80 ± 1.20	11.20 ± 2.96	12.60 ± 1.83

Animals were infused into hypothalamus with Ghrelin (GHRL) or artificial cerebrospinal fluid (ACSF) employing osmotic pumps model 2006 (0.15 µL/h, 42 days). Differential cell counts were gathered counting of at least 20 seminiferous tubules in each slide. Results are expressed as mean ± S.E.M. $n = 5$ animals/group.

Table 4 Ghrelin effect on the morphometry of mice testis chronically treated for 42 days.

	ACSF	**GHRL** (0.3 nmol/day)	**GHRL** (3.0 nmol/day)
Seminiferous tubular diameter (μm)	216.23 ± 7.76	206.23 ± 4.60	208.02 ± 12.28
Seminiferous epithelium height (μm)	66.83 ± 1.47	62.76 ± 3.39	63.68 ± 1.86

Animals were infused with Ghrelin (GHRL) or artificial cerebrospinal fluid (ACSF) employing osmotic pumps model 2006 (0.15 μL per hour, 42 days). Testicular morphometry analyzes were assessed as seminiferous tubular diameter (μm) and the epithelium height (μm) taking into account 50 round or nearly round seminiferous tubules from each mouse. Results are expressed as mean ± S.E.M. $n = 5$ animals/group.

to be more likely related to reduce circulating levels of LH rather than FSH levels.

It is well known that spermatogenesis is a complex process that requires the correct interplay and timing of cellular modifications to produce functional and motile sperm. During this process, spermatogonia undergoes a series of transformations, culminating with the hypercompaction of DNA into the sperm head by replacing histones with a specialized DNA-binding protein, protamine (Kanippayoor *et al.* 2013). Ultrastructural observations of treated animals in our experiments showed an abnormal pattern of chromatin condensation and altered morphology of the acrosome. These alterations could be another possible alternative to explain the changes observed in motility in our research.

On the other hand, hypothalamic GHRL administration for 7 days did not modify spermatogenesis, neither in number nor in functional activity, expressed as motility, viability, response to hypoosmotic swelling test and functionally intact sperm membrane for the correct performance of this gamete. As stated before, epididymal maturation, necessary for the presentation of straight morphology and the acquisition of motility, is an androgen-dependent process (Fernandez *et al.* 2008) and treatment for 7 days was not enough to produce significant changes in testosterone levels, consequently, the quality of the gametes was unaffected.

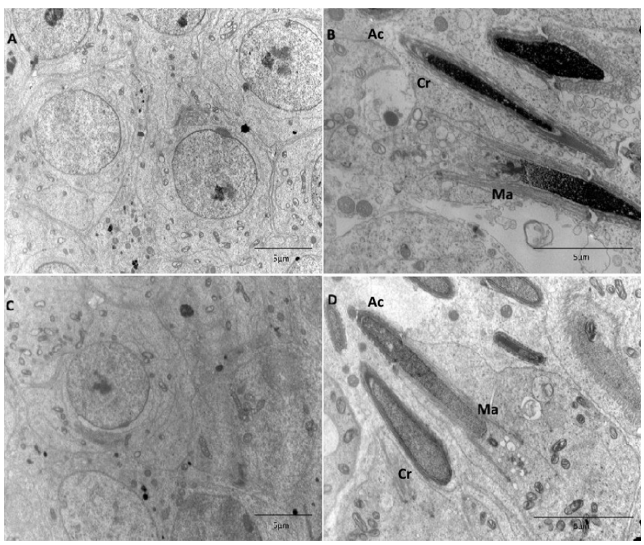

Figure 10 Electron micrographs from seminiferous tubules in mice chronically treated for 42 days. Animals were infused into hypothalamus with Ghrelin (GHRL 3.0 nmol/day – C and D) or artificial cerebrospinal fluid (ACSF, A and B) employing osmotic pumps model 2006 (0.15 μL/h, 42 days). (A and C) Sertoli cells (scale bar: 5 μm). (B and D) Spermatid and spermatozoon (scale bar: 5 μm). Panel D vs B: GHRL-infused animals (D) showed changes in spermatozoa nucleus where chromatin (CR) presented an abnormal condensation pattern; the morphology of the acrosome (Ac) was altered; a large amount of elongated spermatids can be observed, which is evidenced by the presence of a transient structure called the manchette (Ma). Panel C vs A: no morphological differences were observed in Leydig cells. *n*: 3–5 animals/group.

As GHRL is an orexigenic peptide, we daily measured body weight and food intake in order to minimize potential bias of major differences in body weight that could affect reproductive endpoints under analysis. Results presented here confirm that GHRL increases food intake, which was previously demonstrated in some reports (Tschöp *et al.* 2000, Nakazato *et al.* 2001, Wren *et al.* 2001); however, the effect did not last beyond the fifth day of treatment. These results are in line with other authors that have shown that chronic elevation of GHRL in the hypothalamus produces an increase in food intake that leads to an increase in body weight, but this effect seems to be only transient and normalizes after a few weeks (Qi *et al.* 2015). In addition, there are some authors who state that GHRL is not a critical orexigenic factor, based on the observation that mice deficient in either ghrelin or its receptor exhibit normal feeding behavior (Sun *et al.* 2003, 2004, Wortley *et al.* 2004, Sato *et al.* 2008). According to this, chronic effects observed on reproductive parameters cannot be attributed to this variable.

In conclusion, this study provides new evidence about deleterious effect of centrally administered GHRL on functional sperm activity and sex steroids production and suggests that this effect is mediated through hypothalamus by inhibition of HPG axis. However, futures studies are necessary to contribute to the understanding of the molecular mechanisms of GHRL actions.

Declaration of interest

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

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