Inhibitory Effects of Ghrelin on Sexual Behavior: Role of the Peptide in the Receptivity Reduction Induced by Food Restriction in Mice

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Key words
- ghrelin antagonist
- (D-Lys3)-GHRP-6
- sexual behavior
- lordosis
- undernutrition
- food deprivation

Abstract

Ghrelin (Ghr) is a gut/hypothalamus peptide with inhibitory actions on reproductive physiology; however, there are no previous reports of its role on estrous behavior. Under the hypothesis that the increase of plasma Ghr during food restriction (FR) is responsible for receptivity reduction, we intended to evaluate the receptivity percentage of female mice subjected to: exp. 1) acute and chronic FR and Ghr administration (3 nmol/animal/day, s.c.) and exp. 2) the co-administration of a ghrelin antagonist [ant = (D-Lys3)-GHRP-6; 6 nmol/animal/day s.c.]. All females were ovariectomized, primed with steroids, trained, and randomly subjected every week to each one of several protocols, followed by a behavioral test. Experiment 1 (n = 8): basal, no treatment; acute FR (aFR), 24-h fasting; chronic FR (cFR), 50% FR for 5 days; acute ghrelin (aGhr), Ghr 30 min before test and chronic ghrelin (cGhr), Ghr for 5 days. Except for cGhr, all treatments significantly decreased the percentage of receptivity (mean ± SEM): basal 61.9 ± 6.0, aFR 33.1 ± 8.1, cFR 18.8 ± 7.7, aGhr 45.6 ± 10.6, $p < 0.05$ vs. basal. In exp. 2 (n = 11), except for cFR + ant (55.0 ± 6.4) the co-administration of the antagonist reversed the deleterious effects detected in exp. 1: basal 70.9 ± 5.4; aFR + ant 72.3 ± 7.6; aGhr + ant 73.6 ± 4.7. As expected, the administration of vehicle or antagonist alone did not modify receptivity. Besides, we found a significant correlation between percentage of body weight loss and percentage of receptivity reduction ($r = 0.62$, $p = 0.0004$). This is the first study demonstrating that ghrelin is able to inhibit female mice sexual behavior and that is involved, at least in part, in receptivity reduction after food scarcity.

Introduction

The physiological mechanisms that control energy balance are reciprocally linked to those that control reproduction [1]. Therefore, during periods of food scarcity, energy can be conserved by inhibition of unnecessary processes for immediate survival, like hypothalamic-pituitary-gonadal (HPG) axis secretions [2], estrous cyclicity [3], and sexual behavior [1, 4]. This adaptative mechanism, called "nutritional infertility" allows mammals, especially females, to maximize reproductive efficiency and synchronize reproduction with nutritional resources availability [1, 4, 5].

Chronic or acute food restriction decreases female receptivity in several species, including humans [1, 5, 6]. This inhibition reverts promptly after refeeding, even before body weight reaches control values [7–9]. This short-term modulation of sexual behavior has been related to modifications in cellular expression of estrogen receptors (ERs) in the hypothalamic nuclei linked to reproductive behaviors [1, 10, 11].

A major question concerns the nature of the metabolic cues signaling the neural circuits that control sexual behavior about the availability of usable energy [5]. One hypothesis is that the key factor is the short-term availability of oxidizable metabolic fuels, a sign detected in viscera and area postrema. However, little is known about the neuroanatomical pathways and neurotransmitters/neuropeptides that convey metabolic information from detectors to forebrain effectors [5]. The second hypothesis refers to the existence of circulating hormones or peptides that inform the central nervous system about metabolic fuel availability. In this respect, the list of hormones probably responsible for sexual behavior inhibition has grown rapidly and includes insulin, growth hormone, NPY, CRH, and GnRH, among others (reviewed in [1] and [5]).

received 23.02.2011
accepted 27.04.2011

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DOI http://dx.doi.org/10.1055/s-0031-1277228
Horm Metab Res 2011; 43: 494–499

ISSN 0018-5043

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Ghrelin (Ghr), the natural ligand for the growth hormone secretagogue receptor (GHSR) discovered in 1999, is a peptide secreted in the stomach and hypothalamus in response to fasting or undernutrition [12]. This peptide operates as a pleiotrophic modulator of sexual maturation, HPG axis secretions and reproduction, mainly showing inhibitory effects [13–15]. Nonetheless, Ghr has many properties that make it an ideal modulator of sexual behavior: a) its plasma concentration increases significantly in acute or chronic underfeeding protocols, b) after refeeding, Ghr concentration promptly reaches normality (is a "short-term" metabolic modulator), c) the peptide is also secreted in the hypothalamus and systemic ghrelin crosses blood-brain barrier and/or reaches central nervous system via the vagus nerve, and d) Ghr has been reported to modify the circulating and/or central concentrations of several of the hormones mentioned above (GH, NPY, CRH, and GnRH) [12, 16–21]. However, to our knowledge, there are no studies exploring its possible role over female sexual behavior.

Based on the hypothesis that the increase of plasma ghrelin during fasting or food restriction would be responsible for the reduction of female sexual receptivity, the objectives of our study were to evaluate, in 2 consecutive experiments: a) the modifications induced by undernutrition and ghrelin administration (both in acute and chronic mode) upon female mice sexual receptivity and b) the effects of the co-administration of a ghrelin antagonist [(D-Lys3)-GHRP-6] upon female reproductive behavior.

Material and Methods

Animals

Adult female Swiss albino mice (2–3 months of age) were ovariectomized under general anesthesia with a combination of 55 mg/kg of ketamine and 11 mg/kg of xylazine (i.p.). Animals were individually housed in solid-bottom cages (30 cm long × 20 cm wide × 14 cm high) with pine wood shavings as bedding. The room was maintained on a 14:10 h light:dark cycle (lights on at 5 PM). Ambient temperature was 22 ± 2 °C. Rat/mice pelleted food (GEPSA Feeds, Pilar-Córdoba, Argentina) was provided in accordance to the protocols described below and water was available ad libitum. All experiments were conducted in accordance with the Animal Care and Use Guidelines of the Medical School – National University of Cordoba, Argentina.

Experimental design

Three weeks after ovariectomy, females were trained in mating behavioral test employing experienced intact males. During the third week of training, females that exhibited an average sexual receptivity of 50–75% were selected for the experiments (initial basal receptivity). In all cases and in order to achieve receptivity, females were injected with estradiol benzoate (10 µg/animal/day s.c. in 0.1 ml sesame oil, 52 and 28 h before the onset of the behavioral test) and progesterone (500 µg/animal/day s.c. in 0.1 ml sesame oil 4 h before behavioral test) [22].

Ghrelin was purchased from Global Peptides (Fort Collins, CO, USA) and (D-Lys3)-GHRP-6 from Sigma Chemical Co. (St. Louis, MO, USA). Both substances were dissolved in isotonic solution of NaCl (0.9%) and injected subcutaneously in 0.1 or 0.2 ml of solution depending on chronic or acute protocols, respectively.

Experiment 1

For a period of 7 weeks, each female was weekly and randomly subjected to 5 protocols:

- Initial and middle basal: no treatment.
- Acute food restriction (aFR): 24-h fasting before the behavioral test.
- Chronic food restriction (cFR): 50% food restriction for 5 days before the behavioral test. A week after this treatment, females were allowed to obtain food ad libitum for body weight recovery.
- Acute ghrelin (aGhr): 3 nmol/animal of ghrelin (s.c.) 30 min before the behavioral test
- Chronic ghrelin (cGhr): 3 nmol/animal/day of ghrelin (s.c.) for 5 days previous to the behavioral test. Ghr was administered in 2 injections/day of 1.5 nmol each (10 AM and 7 PM).

Experiment 2

Based on the results of experiment 1, a different group of females was weekly and randomly subjected to 6 protocols:

- Initial basal: no treatment.
- Acute food restriction + ghrelin antagonist (aFR + ant): 24-h fasting + 6 nmol/animal/day of (D-Lys3)-GHRP-6 before the behavioral test. The ghrelin antagonist was administered in 2 injections of 3 nmol each, at 10 AM and 7 PM.
- Chronic food restriction + ghrelin antagonist (cFR + ant): 50% food restriction + 6 nmol/animal/day of (D-Lys3)-GHRP-6 for 5 days before the behavioral test. The antagonist was administered in 2 injections of 3 nmol each, at 10 AM and 7 PM. A week after this treatment, females were allowed to obtain food ad libitum for body weight recovery.
- Acute ghrelin + ghrelin antagonist (aGhr + ant): 3 nmol/animal of ghrelin + 6 nmol/animal of (D-Lys3)-GHRP-6, both injected immediately and consecutively 30 min before the behavioral test.
- Isotonic solution: 0.2 ml of isotonic solution 30 min before the behavioral test.
- Ghrelin antagonist: 6 nmol/animal of (D-Lys3)-GHRP-6 30 min before the behavioral test.

During the 8th week and as a positive control, all females of experiment 2 were subjected to a chronic food restriction protocol.

Ghrelin dose

The dose of ghrelin used in the present study was selected from a dose-GH secretion curve previously performed in our laboratory (**Fig. 1**). Ghrelin (0.5, 1, 2, and 3 nmol/animal) was dissolved in isotonic solution (NaCl 0.9%) and injected s.c. to 7–10 animals/dose matched in body weight. After 30 min, mice were sacrificed by decapitation to collect blood. After centrifugation, plasma was stored at −20 °C until hormonal quantification. Plasma GH concentrations were quantified (in duplicate) using a rat/mouse ELISA kit (Millipore, Missouri, USA). Assay sensitivity was 0.07 ng/ml and intra-assay coefficient of variation was 4.0 ± 1.0% (n = 85).

Food and body weight monitoring procedure

Food was weighed and provided freshly each day at 10 AM. Females subjected to basal and isotonic solution protocols were fed ad libitum. Chronically food restricted females received the 50% of the average individual food intake. Ghrelin and (D-Lys3)-GHRP-6 injected females received a full ration in order to avoid an increase in food intake due to the possible orexigenic effects

of ghrelin. Females body weight was evaluated after each behavioral test to calculate the percentage of body weight change after treatment [(final body weight × 100/basal body weight) – 100].

Mating behavior test

All mating behavior tests were conducted between 2 and 3 PM. Females were placed in a clean glass box (30 cm long, 17 cm wide, 20 cm high) for 10 min under red light. After this adaptation period, a sexually experienced male was added into the box. The percentage of female receptivity to the first's 10 mounts/intromissions/ejaculations was evaluated (number of times female was receptive × 100/number of mounts). A female was considered receptive when exerting lordosis (receptive posture with vertebral column dorsiflexion and extension of the legs) or a still posture (female remains motionless, with the legs extended but without column dorsiflexion) were observed [23,24]. If the stimulant male ejaculated before the 10th mount, it was replaced by another.

Statistical analysis

Results were expressed as mean ± SEM. Data from the Ghr/GH curve were analyzed by ANOVA, with LSD Fisher test for post hoc comparisons. Mating behavior tests (experiments 1 and 2) were analyzed by nonparametric repeated measures ANOVA (Friedman). Possible associations between body weight and sexual behavior were evaluated by correlation coefficients (Spearman). In all cases, results were considered significantly different when $p < 0.05$.

Results

The doses of ghrelin that provoked significant rise in plasma GH in comparison to animals injected with saline solution, were 2 and 3 nmol Ghr/animal (**Fig. 1**).

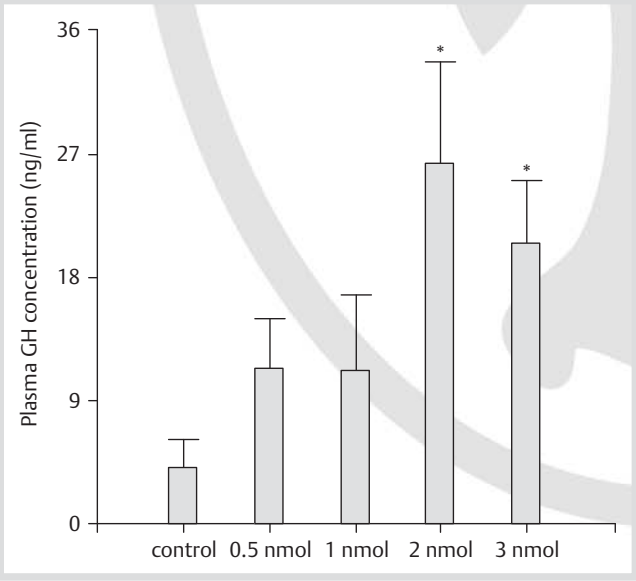

Fig. 1 Plasma GH surge 30 min after the administration to adult male mice (s. c. in 0.1 ml of isotonic solution) of various doses of ghrelin (0.5 nmol n = 8; 1 nmol n = 7; 2 nmol n = 9 and 3 nmol n = 9). Control animals (n = 10) were injected with 0.1 ml of vehicle. Results are expressed by means ± SEM and were analyzed by ANOVA * $p < 0.05$ vs. control.

Regarding the sexual behavior, no differences were noted between initial and middle basal receptivity (68.8 ± 6.0 %, n = 8 and 55.0 ± 9.0 %, respectively, n = 8). Therefore, basal receptivity was estimated based on the average of these 2 values. The higher negative effects upon the percentage of receptivity were detected in females subjected to chronic food restriction (68.3 ± 12.1 % of diminution vs. basal). Receptivity reduction was also found in acute food restriction and after ghrelin (acute and chronic) administration (reductions of 42.5 ± 15.6 %, 24.8 ± 17.9 % and 22.4 ± 14.5 %, respectively). These reductions reached statistical significance vs. basal in all treatments except for chronic Ghr. **Fig. 2** (experiment 1) shows the receptivity percentage of the above mentioned females.

In the second series of experiments (**Fig. 2**, experiment 2), the administration of a Ghr antagonist significantly reversed the detrimental effects exerted by acute food restriction and acute ghrelin injection, reaching basal-like receptivity values. Although the antagonist administration increased receptivity in chronically food restricted females, we still detected a significant reduction in this parameter compared to basal values.

As expected, acute administration of isotonic solution (ghrelin vehicle) or ghrelin antagonist alone did not modify females sexual behavior (isotonic solution: 61.8 ± 6.6 %, n = 11 and (D-Lys3)-GHRP-6: 70.9 ± 7.5 %, n = 11). Once again and as a positive control, in the second series of experiments, chronic food restriction significantly diminished receptivity percentages (data not shown).

With respect to body weight and changes in its values, significant reductions were only found in females subjected to protocols of food restriction (chronic or acute, with or without ghrelin antagonist) (**Table 1**). We did not find a significant correlation between body weight and sexual performance, ($r = 0.24$, $p = 0.16$). Nevertheless, when considering only females that had reduction of their body weight, a highly significant correlation was detected between the percentage of body weight loss (vs. basal) and the percentage of receptivity reduction (vs. basal) ($r = 0.62$, $p = 0.0004$, n = 22).

Discussion

To our knowledge, this is the first study that evaluates the role of ghrelin upon female sexual behavior. Interestingly, we found that this peptide is involved, at least in part, in the receptivity reduction exerted by acute or chronic food restriction in mice. Moreover, the acute administration of ghrelin to well-fed female mice significantly impaired their sexual performance.

In experiment 1, 50 % of chronic food restriction and 24-h fasting significantly diminished female receptivity. In accordance, various protocols of chronic or acute food restriction resulted in a significant reduction of female receptivity in several species, including musk shrews, rat, hamster, and mice. The facts that the above mentioned females were ovariectomized and primed with gonadal steroids indicate that undernutrition alters female sexual behavior, acting directly upon the central nervous system and not through the HPG axis [2,6,11].

We have previously demonstrated that the protocol of 50 % chronic food restriction for 28 days to adult female mice significantly increased plasma ghrelin concentrations from 317.9 ± 82.1 pg/ml in the ad libitum fed animals to 934.6 ± 214.8 pg/ml ($p < 0.05$) in the undernutrited ones [25]. Without omitting the methodological difference in the length of food restriction

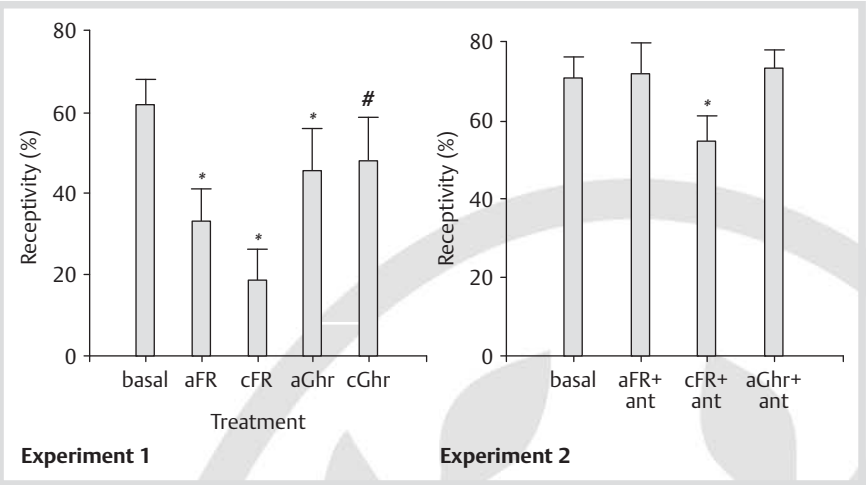

Fig. 2 Sexual receptivity (%) of ovariectomized mice injected with estradiol benzoate and progesterone and subjected randomly to each one of the various treatments during consecutive weeks. Experiment 1: basal = no treatment; aFR = 24-h fasting; cFR = 50% food restriction for 5 days; aGhr = 3 nmol/animal of ghrelin (s.c.) 30 min before behavioral test and cGhr = 3 nmol/animal/day of ghrelin (s.c. in 2 injections/day of 1.5 nmol/each) for 5 days. Experiment 2: basal = no treatment; aFR + ant = 24 h fasting with the injection of 6 nmol/animal (s.c., in 2 injections/day of 3 nmol/each) of (D-Lys3)-GHRP-6, ghrelin antagonist; cFR + ant = 50% food restriction for 5 days with the injection of 6 nmol/animal/day (s.c., in 2 injections/day of 3 nmol/each) of (D-Lys3)-GHRP-6 and aGhr + ant = 3 nmol/animal of ghrelin (s.c.) with the injection of 6 nmol/animal of (D-Lys3)-GHRP-6 (s.c.) 30 min before behavioral test. Results are expressed by means ± SEM and were analyzed by nonparametric repeated measures ANOVA (Friedman). Experiment 1: n = 8; * $p < 0.05$ vs. basal and # $p < 0.05$ vs. cFR. Experiment 2: n = 11; * $p < 0.01$ vs. basal.

Table 1 Body weight and change in body weight in ovariectomized female mice treated with different protocols

Treatment	Body weight after treatment (g)	Change in body weight vs. basal (%)
Experiment 1 (n = 8)		
Basal	35.96 ± 1.23	–
Acute food restriction	32.88 ± 1.38#	−8.52 ± 2.50#
Chronic food restriction	29.76 ± 1.07*	−17.08 ± 2.20*
Acute ghrelin	37.20 ± 0.92	3.73 ± 1.53
Chronic ghrelin	36.59 ± 1.03	2.00 ± 1.95
Experiment 2 (n = 11)		
Basal	35.91 ± 1.09	–
Acute food restriction + ghrelin antagonist	32.88 ± 1.21#	−8.40 ± 2.21#
Chronic food restriction + ghrelin antagonist	30.08 ± 1.00*	−16.09 ± 2.18*
Acute ghrelin + antagonist	37.46 ± 0.85	4.58 ± 1.59

Each female was randomly subjected to each one of the treatments in consecutive weeks. A week after chronic food restriction or chronic food restriction + ghrelin antagonist, no treatment was applied. Body weight was determined after the behavioral test. Basal: no treatment; Acute food restriction: 24-h fasting; Chronic food restriction: 50% food restriction for 5 days; Acute ghrelin: 3 nmol/animal of ghrelin (s.c.) 30 min before behavioral test; Chronic ghrelin: 3 nmol/animal/day of ghrelin (s.c. in 2 injections/day of 1.5 nmol/each) for 5 days; Acute food restriction + ghrelin antagonist: 24-h fasting with the injection of 6 nmol/animal (s.c., in 2 injections/day of 3 nmol/each) of (D-Lys3)-GHRP-6; Chronic food restriction + ghrelin antagonist: 50% food restriction for 5 days with the injection of 6 nmol/animal/day (s.c., in 2 injections/day of 3 nmol/each) of (D-Lys3)-GHRP-6 and Acute ghrelin + antagonist: 3 nmol/animal of ghrelin (s.c.) with the injection of 6 nmol/animal of (D-Lys3)-GHRP-6 (s.c.) 30 min before behavioral test. Results are expressed by means ± SEM and were analyzed by ANOVA. In experiment 1, basal value was calculated as the average between initial and intermediate basal body weight. In experiment 1, * $p < 0.0001$ vs. all the other treatments; # $p < 0.0001$ vs. basal, acute ghrelin and chronic ghrelin. In experiment 2, * $p < 0.0001$ vs. all the other treatments; # $p < 0.0001$ vs. basal and acute ghrelin

between that study and this one, our previous results support the 50% FR as a valid model to evaluate the effects of ghrelin upon sexual behavior. Unfortunately, we were unable to measure plasma ghrelin of the female mice fastened for 24 h or those injected with ghrelin. Nevertheless, the results obtained after the co-administration of the antagonist support an increase of the plasma ghrelin concentration in food restricted and 3 nmol ghrelin injected animals.

The only study that explored the role of ghrelin over the reproductive behavior has been recently published by Shah and Nyby [26]. They found that in males, acute ghrelin injection (in similar doses than those used in our study) suppressed courtship behavior and intermale aggression 20 min after i.p. administration. The authors suggest that this quick inhibition was mediated by direct effects of ghrelin on the brain, rather than through inhibition of the HPG axis [26]. In our study, acute administration of ghrelin (30 min before sexual behavioral test) significantly diminished female sexual receptivity. The fact that in experiment 2 the co-administration of a ghrelin antagonist reversed this deleterious effect strongly supports the hypothesis that ghrelin was responsible for this receptivity impairment. Concordantly, (D-Lys3)-GHRP-6 administration also reversed the deleterious effects exerted by acute food restriction, indicating that the consequent ghrelin rise would be involved in the deleterious effect. Because all our animals were ovariectomized and primed with estradiol and progesterone, an indirect effect of ghrelin via gonadal hormone alteration is unlikely. Nevertheless, one can not discard that the inhibitory effect of food restriction or ghrelin injection upon female receptivity is (also) the consequence of several other events triggered by the enhancement of plasma ghrelin concentrations.

In our experiments, chronic ghrelin administration failed to significantly decrease sexual receptivity. The lack of a deleterious effect may be attributed to the activation of compensatory mechanisms linked to chronic treatments (i.e., downregulation

of GHSR). In a previous study, we found that acute and chronic increases of ghrelin exerted different effects on mice learning and memory, the acute effects stimulant and the chronic ones being inhibitory [25].

Alternatively, the lack of effects could be attributed to the fact that, in our chronic protocol, the last injection of ghrelin was administered 4 h before the behavioral test and with half of the daily dose (1.5 nmol), in comparison to the acute treatment, in which the whole dose was injected 30 min before the test. It has been demonstrated that the s. c. injection of ghrelin resulted in a significant plasma peptide elevation only during the first hour post-injection, consistent with the short half-life of ghrelin [27]. In consequence, it is possible that plasma ghrelin concentrations of chronically treated females at the moment of the behavioral test could be low enough to diminish sexual performance, but not statistically significant.

In the present study, the daily administration of a ghrelin antagonist to chronically food-restricted females reversed, although not completely, the deleterious effects exerted by food deprivation. Possible explanations may be attributed to: a) high endogenous ghrelin concentrations that could not be totally antagonized by the doses of (D-Lys3)-GHRP-6 here employed; b) the fact that the half-life of the antagonist is not long enough to chronically inhibit the endogenous concentration of ghrelin (only for short periods); or c) because chronic food restriction is a stressful treatment. It is well known that chronic or acute food restriction increases plasma corticosterone [28], which in turn inhibits female sexual behavior in mice or rats [29, 30].

Finally, the highly significant correlation between body weight loss and sexual behavior impairment found in our study is consistent with the notion that sexual performance is tightly linked with nutritional status [1, 5].

One limitation of our study was that experiments 1 and 2 were performed in 2 different groups of females. The reason for this design is the fact that after ovariectomy, females may progressively increase their body weight and/or vary their adipose tissue distribution, with the possible rise of plasma leptin concentrations and aromatase activity. Because these factors could modify the sexual responsiveness of females, we chose to limit the number of experimental weeks after ovariectomy to 10–12. The random distribution of protocols selected in our design and the control treatments performed within (i. e., middle basal, cFR in experiment 2, etc.) have the purpose to dilute these possible confusing factors.

As stated before, an important number of hormones and neuropeptides have been proposed as female sexual behavior modulators (GnRH, NPY, CRH, etc) (reviewed in [1] and [5]). Ghrelin is capable to modify their systemic and/or central concentrations; ghrelin infusion in rhesus monkeys pretreated with astressin B (antagonist of the CRH receptor) prevented the plasma LH reduction detected by ghrelin infusion, suggesting that at least at hypothalamus-pituitary physiology, CRH receptor mediates ghrelin LH inhibition [16]. Otherwise, Ghr significantly inhibited in vitro GnRH secretion from hypothalamic fragments of female rats [17] and this peptide is able to reduce the concentrations of kisspeptin, a hormone that upregulates the secretion of GnRH in the preoptic area [18]. Furthermore, hypothalamic and systemic Ghr increases plasma and central NPY concentrations in several species [19, 31, 32].

Altogether, these evidences indicate that ghrelin would be a suitable metabolic modulator of the sexual behavior, acting by different and probably superposed mechanisms. The fact that plasma concentrations of ghrelin increase after food deprivation in significantly higher proportions in females than in males [33], explains the higher susceptibility of this gender to nutritional infertility.

Our results did not allow us to determine whether ghrelin operated via one/more neuropeptide(s) or directly at central levels via its own receptors (GHSR). Further studies are being conducted to explore the effects of i. c. v. ghrelin infusion upon mice sexual performance. Additionally, preliminary results had shown that daily administration of ghrelin antagonist to 50 % chronically food restricted females was capable to reverse the hypothalamic ERα diminution exerted by food deprivation (unpublished results).

In conclusion, to our knowledge this is the first study, which demostrates that ghrelin, a gut secreted hormone, is able to inhibit female mice sexual behavior and is involved in receptivity reduction detected after food deprivation.

Acknowledgements

This study has been supported by grants from the Secretaría de Ciencia y Tecnología, Universidad Nacional de Córdoba (SECyT-UNC), and the Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET). We thank Silvina Gutiérrez Ph.D. and Prof. Alicia Torres Ph.D. from the Centro de Microscopía Electrónica, Universidad Nacional de Córdoba. VPC, RDR, MfdeC, and ACM are established investigators from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Córdoba, Argentina. EML is a fellow from Secretaría de Ciencia y Tecnología – Universidad Nacional de Córdoba (SECyT-UNC), Córdoba, Argentina.

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