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Changes in Hypothalamic KiSS-1 System and Restoration of Pubertal Activation of the Reproductive Axis by Kisspeptin in Undernutrition

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Activation of the gonadotropic axis critically depends on sufficient body energy stores, and conditions of negative energy balance result in lack of puberty onset and reproductive failure. Recently, KiSS-1 gene-derived kisspeptin, signaling through the G protein-coupled receptor 54 (GPR54), has been proven as a pivotal regulator in the control of gonadotropin secretion and puberty. However, the impact of body energy status upon hypothalamic expression and function of this system remains unexplored. In this work, we evaluated the expression of KiSS-1 and GPR54 genes at the hypothalamus as well as the ability of kisspeptin-10 to elicit GnRH and LH secretion in prepubertal rats under short-term fasting. In addition, we monitored the actions of kisspeptin on food intake and the effects of its chronic administration upon puberty onset in undernutrition. Food deprivation induced a concomitant decrease in hypothalamic KiSS-1 and increase in GPR54 mRNA levels in prepubertal rats. In addition, LH responses to kisspeptin *in vivo* were enhanced, and its GnRH secretagogue action *in vitro* was sensitized, under fasting conditions. Central kisspeptin administration failed to change food intake patterns in animals fed *ad libitum* or after a 12-h fast. However, chronic treatment with kisspeptin was able to restore vaginal opening (in ~60%) and to elicit gonadotropin and estrogen responses in a model of undernutrition. In summary, our data are the first to show an interaction between energy status and the hypothalamic KiSS-1 system, which may constitute a target for disruption (and eventual therapeutic intervention) of pubertal development in conditions of negative energy balance. (*Endocrinology* 146: 3917–3925, 2005)

PITUITARY GONADOTROPINS LH and FSH are structurally related glycoproteins responsible for the control of gonadal development and function (1, 2). Secretion of both gonadotropins is primarily driven by the pulsatile release of the hypophysiotropic hypothalamic decapeptide GnRH, also termed GnRH-I (1–5). Full activation of the gonadotropic axis takes place at puberty, which is the culmination of a cascade of developmental events that ultimately leads to enhanced activity of the GnRH pulse generator, increased plasma levels of LH and FSH, and attainment of reproductive capacity (2–5). Awakening of the reproductive axis at puberty, and its subsequent functioning in adulthood, is critically dependent on sufficient body energy stores (5, 6). Indeed, diverse conditions of persistent negative energy balance, such as undernutrition and extreme physical exercise, are associated with lack of puberty onset and reproductive failure, both in humans and experimental animals (6–8). Despite recent developments in the field, including characterization of the pivotal role of leptin in this function, the signals and circuitries responsible for the concerted control of energy balance and reproduction are yet to be totally elucidated (9). Likewise, although decreased pulsatile secretion of GnRH has been ultimately invoked (8), the molecular mechanisms underlying disruption of reproductive function in situations of energy insufficiency remain to be fully characterized.

The master position of hypothalamic GnRH in the hierarchy of signals controlling the gonadotropic axis makes it the target of multiple regulators of central and peripheral origin, and a wide array of excitatory and inhibitory circuits governing GnRH secretion have been identified over the last decades (3–5). In this context, an unsuspected key role for the KiSS-1/G protein-coupled receptor 54 (GPR54) system in the central control of the GnRH-gonadotropin axis has recently arisen on the basis of genetic and pharmacological studies (10). KiSS-1 was originally identified as a metastasis suppressor gene encoding a number of structurally related peptides, generated by the differential proteolytic processing of a common precursor, globally termed kisspeptins (11–13). The biological actions of kisspeptins are conducted through interaction with the previously orphan G protein-coupled receptor GPR54, also termed AXOR12 or hOT7T175 (11–13). A number of point mutations and deletions of the GPR54 gene were recently found in patients suffering idiopathic hypogonadotropic hypogonadism (14–16), a syndrome that was reproduced in mouse models carrying null mutations of the GPR54 gene (15). Thereafter, hypothalamic expression of KiSS-1 and GPR54 genes has been proven as developmen-

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Abbreviations: C_T, Cycle threshold; GALP, galanin-like peptide; GPR54, G protein-coupled receptor 54; icv, intracerebroventricular; NPY, neuropeptide Y.

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tally (maximum at puberty) and hormonally (by sex steroids) regulated (17–19), and the ability of kisspeptins to potently elicit gonadotropin secretion has been simultaneously reported by different groups, including ours (17–26). Moreover, direct stimulatory actions of kisspeptin upon hypothalamic GnRH neurons and/or GnRH release have been very recently documented (18, 19, 23), and mechanistic studies have suggested a relevant role of KiSS-1 signaling in puberty onset in rodent and primate species (19, 22). In fact, comparative meta-analyses with published data on the LH-releasing activity of other neuropeptides and neurotransmitters, such as glutamate and galanin-like peptide, evidence that the KiSS-1 system is likely the most potent elicitor of the GnRH-gonadotropin axis known so far (24, 25). Indeed, although evaluation of its potential interplay with other well known modulators of the reproductive system has been initiated only recently (24, 25), the KiSS-1 system has been already proposed as an essential gatekeeper for proper function of the gonadotropic axis (15, 19).

In the above scenario, we hypothesized that the mechanisms whereby conditions of negative energy balance hamper the functioning of reproductive axis may target directly the hypothalamic KiSS-1 system. To test this hypothesis, expression analyses of KiSS-1 and GPR54 genes at the hypothalamus were conducted, and reproductive responses to kisspeptin (in terms of hormone secretion and biomarkers of puberty onset) were monitored at puberty in models of severe undernutrition.

Materials and Methods

Animals and drugs

Wistar rats bred in the vivarium of the University of Córdoba were used. The day the litters were born was considered as d 1 of age. The animals were maintained under constant conditions of light (14 h of light, from 0700 h) and temperature (22 C), and were weaned at 21 d of age in groups of five rats per cage with free access to pelleted food and tap water, unless otherwise stated. Experimental procedures were approved by the Córdoba University Ethical Committee for animal experimentation and were conducted in accordance with the European Union normative for care and use of experimental animals. The animals were humanely killed by decapitation at the end of the experimental settings. Mouse KiSS-1 (110–119)-NH_2, the rodent analog of the C-terminal KiSS-1 decapeptide KiSS-1 (112–121)-NH_2, was obtained from Phoenix Pharmaceuticals Ltd. (Belmont, CA). This peptide fragment, which has been previously shown to maximally bind and activate GPR54 in transfected CHO cells (12, 13), will be referred hereafter as kisspeptin-10.

Experimental designs

In experiment 1, the effects of conditions of negative energy balance on the expression of the KiSS-1 system at the hypothalamus were monitored in prepubertal animals. This stage of postnatal maturation was selected given the well known dependence of normal pubertal development on sufficient energy stores (3). Groups of male and female rats (30 d old; n = 10–12 per group) were subjected to a period of 72 h of fasting (only access to tap water); age-matched rats fed *ad libitum* served as controls. At the end of the fasting period, the animals were killed by decapitation, and the hypothalamus was immediately dissected out, as described in detail elsewhere (17), by a horizontal cut of about 2 mm depth with the following limits: 1 mm anteriorly from the optic chiasm (to include the preoptic area), the posterior border of mamillary bodies, and the hypothalamic fissures. Hypothalamic samples were frozen in liquid nitrogen and stored at −80 C until processing for RNA analysis.

In experiment 2, the functionality of the KiSS-1 system, in terms of gonadotropic responses, was tested under conditions of severe energy deficit. To this end, the ability of kisspeptin-10 to centrally elicit LH secretion was assessed in prepubertal male and female rats (30 d old; n = 10–12 per group) previously subjected to food deprivation for 72 h. A protocol of intracerebroventricular (icv) administration of 1 nmol kisspeptin-10 was carried out, as described elsewhere (17, 22, 24, 25). To allow delivery of kisspeptin into the lateral cerebral ventricle, the animals were implanted with icv cannulae lowered to a depth of 3 mm beneath the surface of the skull; the insert point was 1 mm posterior and 1.2 mm lateral to bregma. A dose of 1 nmol kisspeptin in 10 μl per rat was selected on the basis of our recent data on the ability of this dose to potently elicit LH secretion in rats fed *ad libitum* (17, 24). Trunk blood samples were collected upon decapitation at 15 min after kisspeptin injection. Animals injected with vehicle (NaCl 0.9%) served as controls.

In experiment 3, the central mechanisms for the effects of kisspeptin upon gonadotropin secretion in situations of negative energy balance were explored. To this end, the ability of kisspeptin-10 to elicit GnRH secretion was tested using a static incubation system and hypothalamic fragments from prepubertal female rats (n = 10–12 hypothalamic samples per group) at two different prevailing metabolic states: feeding *ad libitum* and 72 h of fasting. Upon decapitation of the animals, hypothalamic fragments were excised following the tissue limits indicated in experiment 2 and placed into individual incubation chambers containing 250 μl of phenol red-free DMEM for 30 min of preincubation, using a Dubnoff incubator at 37 C with constant shaking (60 cycles/min), under an atmosphere of 95% O_2/5% CO_2. After this period, preincubation media were replaced by DMEM alone or medium containing increasing concentrations of kisspeptin-10 (10^{-12}, 10^{-10}, 10^{-8}, and 10^{-6} M). Incubations were terminated after 30 min, when media were boiled to inactivate endogenous protease activity, and kept at −80 C until assayed for GnRH levels.

In experiment 4, the effects of kisspeptin upon food intake pattern were studied. To this end, groups of male rats (n = 8 per group) were implanted with icv cannulae, and daily intracerebral injection of 1 nmol kisspeptin was conducted at early light phase (0900 h) for 7 consecutive days. As control for the experimental procedure, an additional group of animals (n = 8) were icv injected with 1 nmol ghrelin, a proven orexigenic factor (9). To explore the impact of the prevailing energy status upon the effects of kisspeptin on food intake, the animals were initially allowed to have free access to chow during the first 4 d, and cumulative food intake was monitored at 1.5, 3, 6, 12, and 24 h after each kisspeptin injection. Thereafter, the animals were subjected to 12 h of fasting during the dark phase preceding kisspeptin injection for the last 3 d of treatment, and cumulative food intake was monitored at 1.5, 3, 6, and 12 h after injection.

Finally, in experiment 5, the effects of chronic central administration of kisspeptin-10 on puberty onset in immature female rats under severe undernutrition were monitored. A protocol of 30% restriction in daily food intake *vs.* age-matched control females (fed *ad libitum*) was initiated on d 23 postpartum. Daily icv administration of kisspeptin in the lateral cerebral ventricle was conducted between d 30–37 postpartum in food-restricted females (n = 12), as described in detail elsewhere (22). The treatment regimen was set at a dose of 1 nmol KiSS-1 per animal in 10 μl, every 12 h. Pair-aged females (n = 10), at 30% food restriction, injected with vehicle served as controls. The animals were icv injected under conscious conditions after careful handling to avoid any stressful influence, in keeping with our previous references (22). In all experimental animals, body weight and vaginal opening were daily monitored. On the latter, detailed inspection was conducted in each animal to determine the date of complete canalization of the vagina. At the end of treatment (37 d postpartum), the animals were killed by decapitation, 60 min after the last injection of kisspeptin or vehicle, and trunk blood was collected. For determination of the normal date of vaginal opening in animals fed *ad libitum*, an additional group of females (n = 20), without food restriction and icv injected with vehicle, were maintained on daily inspection of canalization of the vagina up to d 37 postpartum.

RNA analysis by semiquantitative RT-PCR

Total RNA was isolated from hypothalamic samples using the single-step, acid guanidinium thiocyanate-phenol-chloroform extraction method. Hypothalamic expression of KiSS-1 and GPR54 mRNAs was assessed by RT-PCR, optimized for semiquantitative detection, using

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previously defined primer pairs and conditions (17). As internal control for RT and reaction efficiency, amplification of a 240-bp fragment of S11 ribosomal protein mRNA was carried out in parallel in each sample (17). PCR consisted of a first denaturing cycle at 97 C for 5 min, followed by a variable number of cycles of amplification defined by denaturation at 96 C for 30 sec, annealing for 30 sec, and extension at 72 C for 1 min. A final extension cycle of 72 C for 15 min was included. Annealing temperature was adjusted for each target and primer pair: 62.5 C for KiSS-1, 63.5 C for GPR54, and 58 C for RP-S11 transcripts. In keeping with previous optimization tests (17), 32 and 24 PCR cycles were chosen for semiquantitative analysis of specific targets (KiSS-1 and GPR54) and RP-S11 internal control, respectively. Specificity of PCR products was confirmed by direct sequencing (Central Sequencing Service, University of Córdoba, Córdoba, Spain). Quantification of intensity of RT-PCR signals was carried out by densitometric scanning using an image analysis system (1-D Manager; TDI Ltd., Madrid, Spain), and values of the specific targets were normalized to those of internal controls to express arbitrary units of relative expression. In all assays, liquid controls and reactions without RT resulted in negative amplification.

RNA analysis by real-time RT-PCR

To verify changes in gene expression, real-time RT-PCR was performed in the experimental samples using the iCycler iQ Real-Time PCR detection system (Bio-Rad Laboratories, Hercules, CA). In detail, KiSS-1 and GPR54 mRNA levels were assayed in hypothalamic samples from prepubertal male and female rats subjected to 72 h of fasting and their respective controls fed *ad libitum*. General procedures for real-time RT-PCR were as previously described (17). The synthesized cDNAs were further amplified in triplicate by PCR using SYBR green I as fluorescent dye and 1× iQ Supermix containing 50 mM KCl, 20 mM Tris-HCl, 0.2 mM dNTPs, 3 mM Mg Cl_2, and 2.5 U iTaq DNA polymerase (Bio-Rad), in a final volume of 25 μl. The PCR cycling conditions were as follows: initial denaturation and enzyme activation at 95 C for 5 min, followed by 40 cycles of denaturation at 95 C for 15 sec, annealing at 62.5 C (KiSS-1), 63.5 C (GPR54), or 58 C (RP-S11) for 15 sec, and extension at 72 C for 1 min. Calculation of relative expression levels of the target mRNAs was conducted based on the cycle threshold (C_T) method (27). The C_T for each sample was calculated using the iCycler iQ Real-Time PCR detection system software with an automatic fluorescence threshold (R_n) setting. Accordingly, fold expression of target mRNAs over reference values was calculated by the equation $2^{-\Delta\Delta CT}$, where ΔC_T is determined by subtracting the corresponding RP-S11 C_T value (internal control) from the specific C_T of the target (KiSS-1 or GPR54), and $\Delta\Delta C_T$ is obtained by subtracting the ΔC_T of each experimental sample from that of the reference sample (taken as reference value 100). No significant differences in C_T values were observed for RP-S11 between the treatment groups.

Hormone measurement by specific RIAs

Serum LH and FSH levels were determined in a volume of 25–50 μl using a double-antibody method and RIA kits kindly supplied by the NIH (Dr. A. F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Peptide Program, Torrance, CA). Rat LH-I-9 and FSH-I-9 were labeled with ^{125}I by the chloramine-T method, and the hormone concentrations were expressed using the reference preparation LH-RP-3 and FSH-RP2 as standards. Intra- and interassay coefficients of variation were less than 8 and 10% for LH, and 6 and 9% for FSH, respectively. The sensitivity of the assay was 5 pg/tube for LH and 20 pg/tube for FSH. In addition, in selected serum samples (experiment 5), serum estradiol levels were determined using a commercial kit from MP Biomedicals (Costa Mesa, CA), following the instructions of the manufacturer. The sensitivity of the assay was 0.5 pg/tube, and the intraassay coefficient of variation was less than 5%. Finally, GnRH levels in incubation media (experiment 3) were assayed using a commercial RIA kit (Peninsula Laboratories, San Carlos, CA), following the instructions of the manufacturer. The sensitivity of the assay was 1 pg/tube. For each hormone, all the samples were measured in the same assay.

Presentation of data and statistics

Hormonal determinations (LH, FSH, and estradiol in serum and GnRH in incubation medium) were conducted in duplicate, with a minimal total number of 10 samples per group. Semiquantitative RT-PCR analyses were carried out in duplicate from at least four independent RNA samples of each experimental group. Quantitative RNA and hormonal data are presented as mean ± SEM. Results were analyzed for statistically significant differences using Student's *t* test or ANOVA followed by Student-Newman-Keuls multiple range test (SigmaStat 2.0, Jandel Corp., San Rafael, CA). $P \leq 0.05$ was considered significant. When appropriate (see experiment 3), the mean effective dose (ED_{50}), defined as the dose of KiSS-1 peptide able to induce 50% of the maximal response, was determined by nonlinear regression (SigmaStat 2.0).

Results

Effects of severe undernutrition upon hypothalamic expression of the KiSS-1 system

To evaluate the effects of situations of negative energy balance on the hypothalamic expression of the KiSS-1 system, relative levels of KiSS-1 and GPR54 mRNAs were assayed by means of final-time and real-time RT-PCR in whole hypothalamic fragments from prepubertal male and female rats after 72 h of fasting. Food deprivation in prepubertal (30 d old) females induced a significant decrease in hypothalamic KiSS-1 mRNA levels that was associated with a moderate but significant increase in GPR54 mRNA expression levels (Fig. 1A). Likewise, a concomitant decrease in KiSS-1 mRNA and increase in GPR54 mRNA levels was observed in hypothalamic samples from prepubertal (30 d old) male rats subjected to 72 h of fasting (Fig. 1B).

Gonadotropic responses to kisspeptin under severe undernutrition

The functionality of the central KiSS-1 system in situations of negative energy balance was explored by assessing the LH- and GnRH-releasing effects of kisspeptin-10 using *in vivo* and *in vitro* models, respectively. For the analysis of LH responses to kisspeptin *in vivo*, prepubertal (30 d old) male and female rats were subjected to food deprivation for 72 h, a procedure that induced a significant reduction in body weight (females, 64.7 ± 1.9 g in fasting *vs.* 77.5 ± 2.5 g in controls; males, 67.0 ± 2.1 g in fasting *vs.* 81.7 ± 2.9 g in controls) and serum LH levels (Fig. 2). In age-matched rats fed *ad libitum*, intracerebral injection of 1 nmol kisspeptin-10 induced robust LH secretory responses, at 15 min after injection, both in females (mean response, ~9.7-fold increase over vehicle-injected animals) and males (mean response, ~9.0-fold increase over vehicle-injected animals). In fasted animals, the ability of similar doses (1 nmol/rat icv) of kisspeptin-10 to elicit LH secretion *in vivo* was preserved. Moreover, the effectiveness of kisspeptin in terms of induction of LH secretion was notably enhanced, as estimated by the absolute LH levels reached at 15 min after injection of the peptide (significantly higher in fasted animals than in controls) as well as by the relative fold increase in serum LH concentrations induced by kisspeptin over control levels in fasted animals injected with vehicle (females, ~62.5-fold increase; males, ~53.5-fold increase) (Fig. 2).

In addition, the ability of increasing doses of kisspeptin-10 (10^{-12} to 10^{-6} M) to elicit the release of GnRH in conditions of negative energy balance was assessed *in vitro* using static incubations of hypothalamic fragments from prepubertal female rats, either fed *ad libitum* or after 72 h of fasting. Kisspep-

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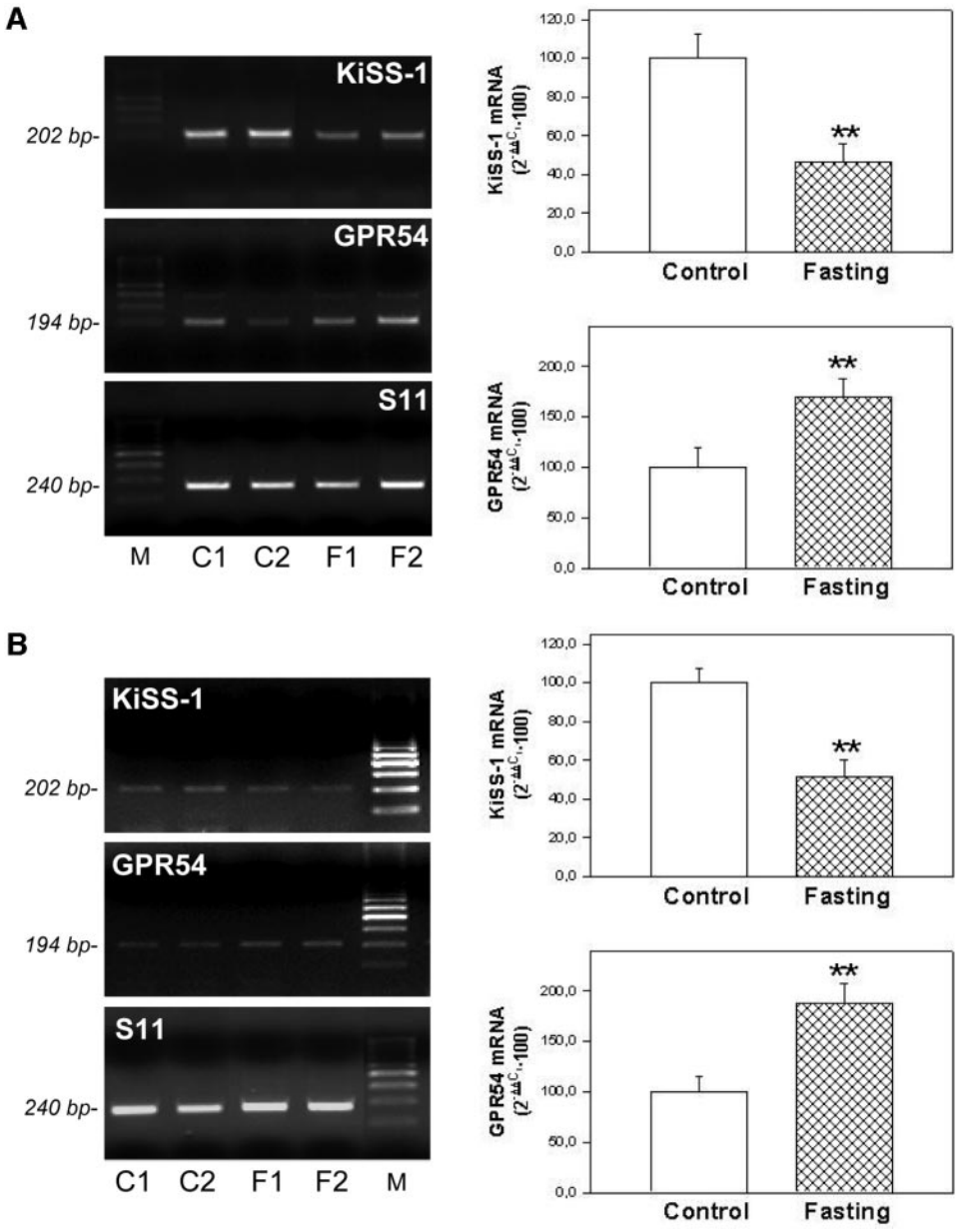

FIG. 1. Effects of short-term (72-h) fasting upon the expression levels of KiSS-1 and GPR54 mRNAs at the hypothalamus in prepubertal (30 d old) female (A) and male (B) rats. In the *left panels*, representative final-time RT-PCR assays, optimized for semiquantitative analysis, of samples from control animals fed *ad libitum* (C) and fasted rats (F) are presented. Two independent samples per group are presented. Parallel amplification of S-11 ribosomal protein mRNA served as internal control. In the *right panels*, expression levels of the targets in the experimental groups, obtained by real-time RT-PCR as described in *Materials and Methods*, are shown. RT-PCR analyses were carried out in duplicate from at least four independent RNA samples, generated by pooling of two to three individual hypothalamic fragments, of each experimental group.**, $P < 0.01$ *vs.* control groups fed *ad libitum* (nonpaired Student's *t* test).

tin-10 was able to dose-dependently stimulate GnRH release by hypothalamic tissue from fed animals, with a threshold no-observed effect level of 10^{-10} M, a predicted ED_{50} of approximately 10^{-9} M, and a maximal response of ~2.6-fold increase over control values (Fig. 3). It is to be noted that such doses are in good agreement with the mean effective and threshold values recently reported by our group for the stimulatory effect of kisspeptin upon LH secretion *in vivo* (24). Fasting for 72 h induced a significant decrease ($P \leq 0.01$) in basal release of GnRH *in vitro*. However, the ability of kisspeptin-10 to dose-dependently elicit GnRH secretion was preserved after food deprivation. Moreover, in this model, the sensitivity to kisspeptin in terms of induction of GnRH secretion was significantly enhanced, as estimated by the lower NOEL (10^{-12} M) and predicted ED_{50} value (~10^{-10} M). In addition, maximal relative responses to kisspeptin over corresponding control values (~4.0-fold) were increased in fasted animals (Fig. 3).

Effects of kisspeptin upon food intake patterns in rats fed ad libitum *and after prefasting*

Considering the impact of body energy status upon the expression and function of the KiSS-1 system, the effects of repeated intracerebral injections of kisspeptin-10 upon food intake were explored. To this end, 1 nmol kisspeptin was daily injected into male rats at the early light phase (0900 h), and cumulative food intake was recorded at different time intervals over a 7-d period. The potential effects of kisspeptin were tested against two different prevailing nutritional states: rats fed *ad libitum* without food restriction (during the first 4 d of the experiment) and rats subjected to a period of

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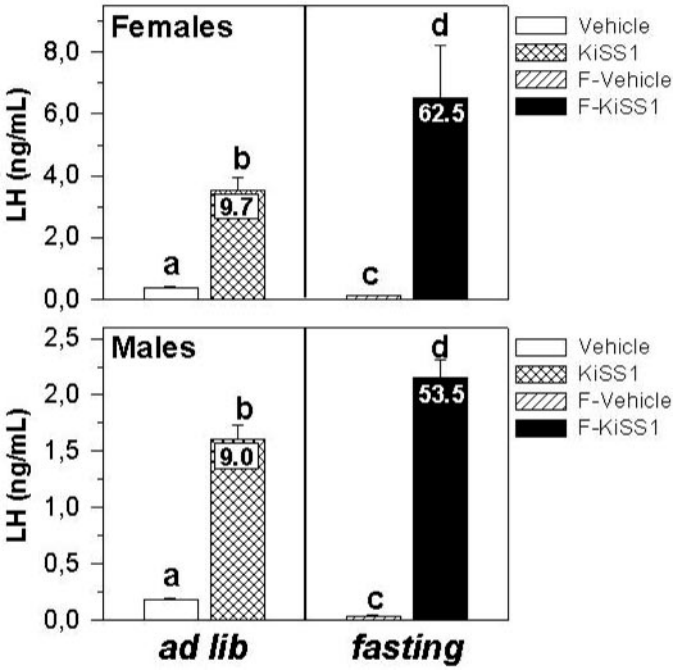

FIG. 2. Effects of central administration of kisspeptin-10 (1 nmol/rat) upon serum LH levels in prepubertal (30 d old) female and male rats, either fed *ad libitum* or subjected to 72 h of fasting. In addition to absolute LH levels, mean relative increases over respective control values injected with vehicle are shown in the *insets*. Hormonal levels are the mean ± SEM of 10–12 determinations per group. Data points with *different letters* are statistically different (ANOVA followed by Student-Newman-Keuls multiple range test).

12 h of food deprivation during the dark phase before injection of kisspeptin (for the last 3 d). In these two models, kisspeptin was unable to significantly modify the pattern of food intake *vs.* vehicle-injected controls. For illustrative purposes, the patterns of cumulative food intake on d 1 and 7 of treatment are shown in Fig. 4. As positive control for the experimental procedure in this setting, icv injection of 1 nmol ghrelin evoked significant orexigenic responses throughout the study period. For example, on d 1 of treatment, intracerebral administration of ghrelin acutely increase food intake (from 0.7 ± 0.4 g in control animals to 1.91 ± 0.45 in ghrelin-treated rats at 1.5 h after injection; $P \leq 0.01$), in keeping with previous references (9).

Effects of KiSS-1 administration on puberty onset under severe undernutrition

The effects of KiSS-1 upon the activation of the reproductive axis were monitored in immature female rats subjected to a protocol of 30% restriction in daily food intake, initiated on d 23 postpartum. A model of repeated intracerebral injection of kisspeptin, starting at d 30 postpartum, was selected to specifically target central (*e.g.* hypothalamic) actions of KiSS-1. Yet, this prevented us from extending the treatment protocol beyond 37 d postpartum (*i.e.* 13 injections of KiSS-1 over 7 d) to avoid misadministration of kisspeptin caused by displacement of the cannulae that takes place (approximately) 7–8 d after their surgical implantation. As an external index of puberty onset, vaginal opening (defined as complete canalization of the vagina) was daily monitored. Such a marker was selected because it has been conventionally considered a reliable external sign of puberty, allowing recapitulation of proper functional activation of all the levels of the reproductive axis (Ref. 22 and references therein). For reference purposes, control reference females fed *ad libitum* were also daily monitored, a group that showed complete vaginal opening at a mean age of 34.9 ± 0.25 d postpartum and mean body weight of 98.5 ± 2.1 g at 37 d postpartum. Chronic food restriction induced a significant reduction in body weight of approximately 30% from controls (mean body wieght, 68.4 ± 1.0 g; n = 22). This regimen totally prevented normal puberty onset because none of the vehicle-injected females in undernutrition presented vaginal opening by 37 d postpartum (mean body weight, 69.3 ± 0.95 g; n = 10). In addition, significantly suppressed basal LH and estradiol levels were detected in food-restricted animals *vs.* fed controls. Likewise, serum FSH levels were significantly lower in underfed females (2.48 ± 0.3 ng/ml *vs.* 5.05 ± 0.21 ng/ml in controls). In contrast, chronic icv administration of kisspeptin-10 (1 nmol/12 h) between d 30 and 37 to females with undernutrition induced complete vaginal opening in seven of 12 treated animals (~60%), with a mean body weight of 67.6 ± 1.0 g. As additional indices of rescue of pubertal activation of the reproductive axis, serum LH and estradiol levels were significantly increased in every single food-restricted female rat after repeated icv injection of kisspeptin (Fig. 5). Similarly, FSH levels in kisspeptin-treated females were significantly higher than in underfed animals injected with vehicle (5.2 ± 0.77 ng/ml *vs.* 2.48 ± 0.3 ng/ml).

Discussion

Activation of the reproductive axis at puberty, and its subsequent functioning in adulthood, critically depends on sufficient energy stores (6–8). It is assumed that the mechanism whereby situations of energy deficit ultimately impair fertility involves a decrease in the activity of the hypothalamic GnRH pulse generator (8). A conspicuous feature, however, of this phenomenon is that GnRH gene expression at the hypothalamus is not apparently reduced in fasting conditions (28). This suggests that central modulation of the GnRH-gonadotropic axis by the energy status and metabolic factors likely takes place at the level of upstream regulatory signals (involved in the control of GnRH release), whose nature is yet to be fully determined. Among others, a pivotal role for the KiSS-1 system in the central control of the reproductive axis has very recently arisen (10). Indeed, different reports have now provided compelling evidence on the ability of kisspeptins to directly activate GnRH neurons and to elicit GnRH secretion (18, 19, 23) (present results). Yet, despite the proposed function of the KiSS-1 system as a major gatekeeper of GnRH neurons (15, 19), its potential involvement in conveying the impact of (insufficient) body energy stores onto the reproductive axis remains to be determined. To our knowledge, the present study provides the first comprehensive mechanistic overview of the interaction between energy status and the KiSS-1 system in the central control of puberty onset and gonadotropin secretion.

The experimental data presented herein evidence that sit-

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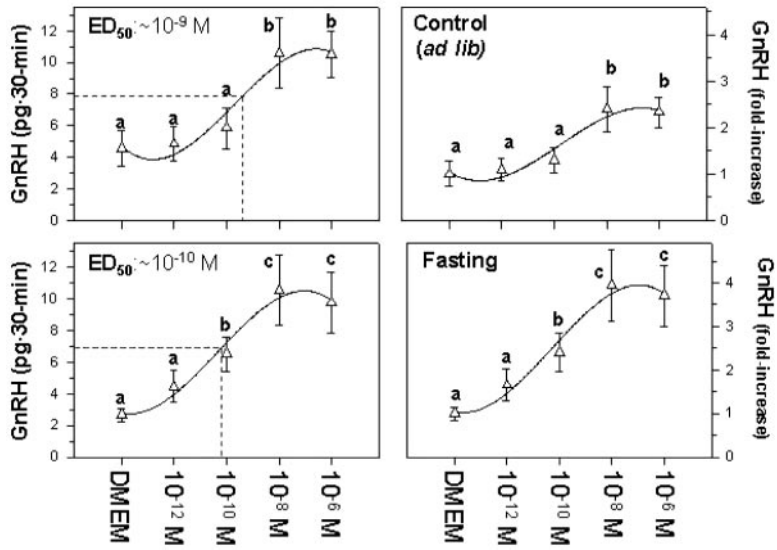

FIG. 3. Effects of increasing doses of kisspeptin-10 on GnRH release by incubated hypothalamic fragments from prepubertal (30 d old) female rats, either fed *ad libitum* (*top*) or subjected to 72 h of fasting (*bottom*). In addition to absolute GnRH levels in the incubation media, relative increases (in terms of fold increase) over respective control values are shown. Hormonal levels are the mean ± SEM of 10–12 determinations per group. Data points with *different letters* are statistically different (ANOVA followed by Student-Newman-Keuls multiple range test).

uations of negative energy balance induce a decrease in the hypothalamic expression of kisspeptin, as suggested by the significant reduction in the relative levels of KiSS-1 mRNA observed in prepubertal male and female rats under short-term fasting. Considering its physiological role in the control of GnRH neurons, it is reasonable to predict that reduced KiSS-1 signaling might operate as a major contributing factor for the reproductive failure (*i.e.* hypogonadotropic hypogonadism) induced by food deprivation. Additional evidence for alterations in the KiSS-1 system in situations of energy deficit comes from the observation of increased GPR54 mRNA levels at the hypothalamus in fasted animals. A tempting explanation for such a paradoxical finding is that a primary decrease in ligand (KiSS-1) expression might bring about a compensatory increase in the expression of its putative receptor (GPR54), inducing a state of sensitization to the effects of kisspeptin. Indeed, this is apparently the case, as evidenced by our *in vivo* and *in vitro* data in short-term fasting. Thus, LH responses to kisspeptin *in vivo* were preserved, and even enhanced, in food-deprived animals, suggesting that replacement of endogenous KiSS-1 levels is sufficient to fully activate the GnRH-LH axis despite adverse metabolic conditions. Likewise, the sensitivity to kisspeptin in terms of induction of GnRH secretion *in vitro* was significantly enhanced (as evidenced by lower mean and minimal effective doses), and its maximal responses increased, in fasted animals. On the former, it has to be noted that 10^{-10} M kisspeptin was able to elicit GnRH release *in vitro* in underfed animals but not in those fed *ad libitum*, which support the contention that the sensitivity to kisspeptin is significantly increased in situations of negative energy balance. Interestingly, in our setting, basal release of GnRH was significantly reduced (~2.0-fold) by previous short-term fasting. Such a decrease, however, was fully reversed by incubation of hypothalamic fragments from fasted animals in the presence of 10^{-10} M kisspeptin (see Fig. 3). This suggests that the physiological level of hypothalamic kisspeptin may range between 10 and 100 pM and that its replacement in conditions of negative energy balance is apparently sufficient to restore normal GnRH release.

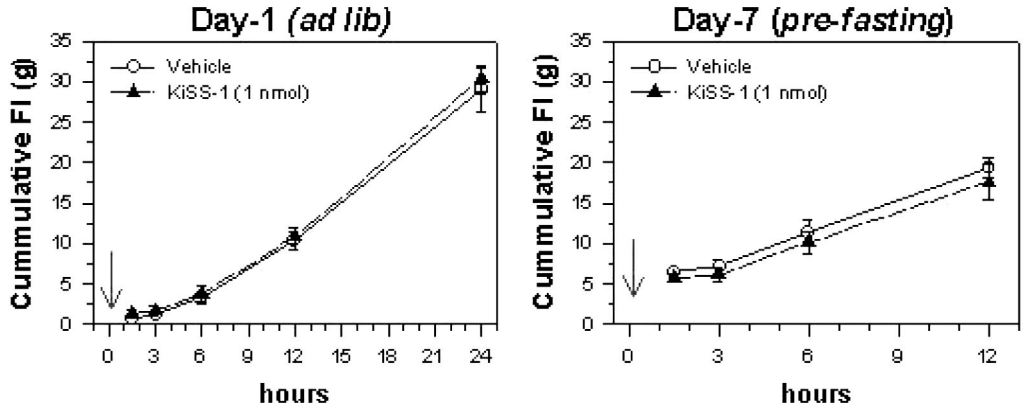

FIG. 4. Effects of kisspeptin-10 upon food intake pattern in rats. A protocol of daily injection of kisspeptin-10 (1 nmol/rat) or vehicle at the early light phase (0900 h) was implemented (n = 8 animals per group), and cumulative food intake was recorded at different time intervals over a 7-d period. Two different prevailing nutritional states were studied: rats fed *ad libitum* (first 4 d of the experiment) and rats subjected to a period of 12 h of food deprivation during the dark phase before injection of kisspeptin (last 3 d). Representative patterns of cumulative food intake on d 1 and 7 of treatment are shown. No significant differences were found between the treatment groups (ANOVA followed by Student-Newman-Keuls multiple range test).

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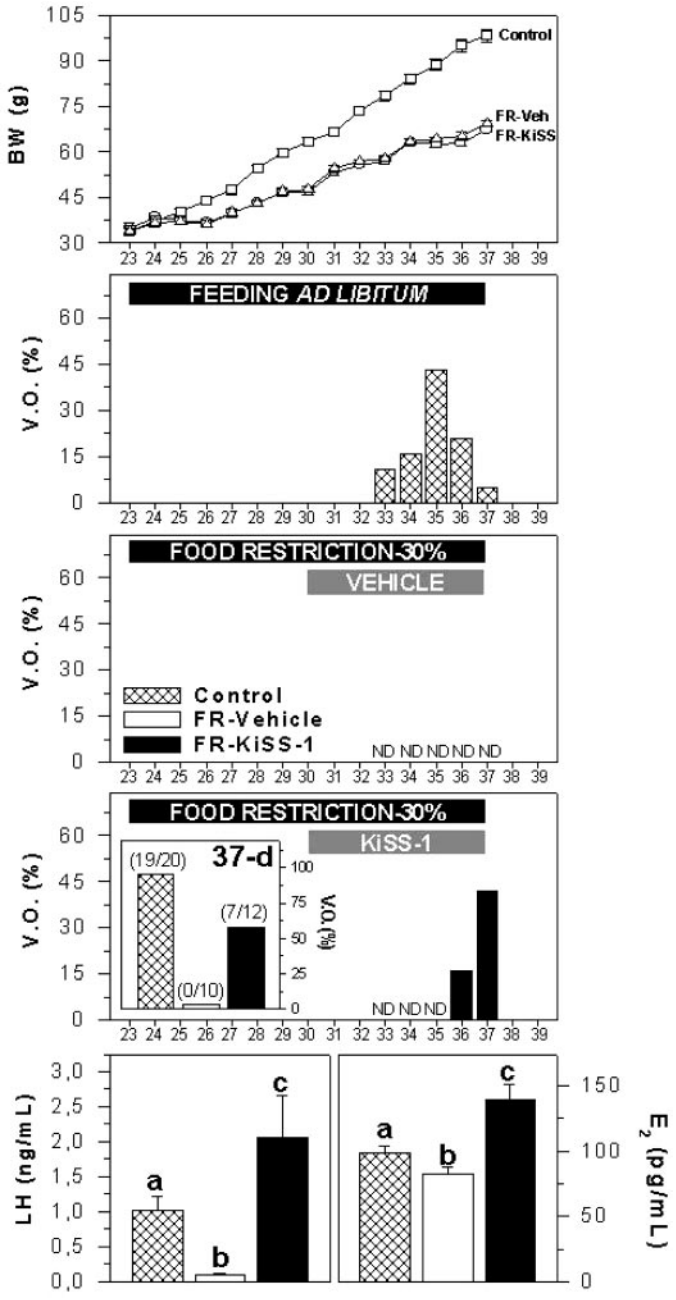

FIG. 5. Compilation of indices of pubertal maturation recorded in peripubertal female rats subjected to a protocol of 30% restriction in daily food intake (FR; 70% food intake of controls), and chronically icv injected with kisspeptin-10 (1 nmol/12 h; n = 12 animals) or vehicle (n = 10 animals) between d 30 and 37. For reference purposes, data from control females (n = 20 animals), fed *ad libitum*, injected with vehicle are also shown. Body weight records in the different experimental groups are presented in the *upper panel*. Dates of vaginal opening (V.O.), expressed as percentage over total number of animals per each experimental group, are shown in the *middle panels*. To note, although undernutrition prevented V.O. in all vehicle-injected animals, kisspeptin administration was able to restore canalization of the vagina in approximately 60% of cases (*inset*). In addition, kisspeptin treatment fully reversed the undernutrition-induced decrease in serum LH levels and significantly elevated serum estradiol concentrations (see *lower panels*). Data points with *different letters* are statistically different (ANOVA followed by Student-Newman-Keuls multiple range test). ND, Not detected (*i.e.* lack of V.O.).

Assumably, the molecular basis for the observed changes in the expression and function of the KiSS-1 system in fasting conditions is yet to be elucidated, but in principle, different signals with proven actions in the joint control of energy balance and reproduction might be involved. Among others, the adipocyte hormone leptin has been identified as a pivotal peripheral factor for signaling the amount of body energy stores to the hypothalamic centers governing reproduction. However, the ultimate mechanisms whereby leptin conducts this relevant function are still a matter of debate, and direct or indirect effects of leptin upon GnRH neurons have been proposed (8). Interestingly, leptin has been involved in the control of the hypothalamic expression of galanin-like peptide (GALP), another potent elicitor of GnRH-LH secretion, and a sensitization phenomenon, analogous to that reported herein for KiSS-1, has been recently described for the LH-releasing effects of GALP in conditions of leptin insufficiency (29). Indeed, hyperresponsiveness to a number of elicitors of the hypothalamic-pituitary-gonadal axis is apparently detected in conditions of negative energy balance and/or hypoleptinemia, a phenomenon where the potential contribution of altered KiSS-1 functions (as a relevant downstream regulator of the GnRH system) merits further investigation. Nonetheless, whether similar mechanisms underlie the reported sensitization to kisspeptin and GALP is yet to be determined. In fact, our preliminary observations evidence that, in contrast to GALP, KiSS-1 mRNA levels at the hypothalamus are not significantly modified in conditions of leptin deficiency (*i.e. ob/ob* mouse) (our unpublished data), suggesting that leptin is not an essential regulator of KiSS-1 gene expression at the hypothalamus. Alternatively, leptin modulation of the KiSS-1 system may take place at a posttranscriptional step, and/or additional neuroendocrine integrators might be involved. The latter possibility is presently under investigation at our laboratory.

Expression of KiSS-1 and GPR54 mRNAs, as well as metastin-like immunoreactivity, has been detected in several hypothalamic areas involved in feeding regulation (21, 30, 31), including the arcuate nucleus. This area has been highlighted as a major center for the integrated regulation of food intake, where neuropeptide Y (NPY)/Agouti-related peptide (orexigenic) and proopiomelanocortin/cocaine- and amphetamine-regulated transcript (anorexigenic) neurons reciprocally operate under the regulation of peripheral factors, such as leptin (29 and references therein). Our present results document, however, that central injection of kisspeptin is not able to significantly alter the pattern of food intake, either in rats fed *ad libitum* or subjected to previous 12 h of fasting. In good agreement, intracerebral administration of kisspeptin, at a dose effective to maximally elicit LH secretion, failed to change hypothalamic expression levels of NPY, Agouti-related peptide, proopiomelanocortin, and cocaine- and amphetamine-regulated transcript mRNAs (our unpublished data). Altogether, our current results demonstrate that, although body energy stores impact the expression and function of the KiSS-1 system at the hypothalamus, kisspeptin is not provided with specific regulatory actions upon feeding. In fact, during conduction of this study, this contention was further supported by an independent study (23). This finding is contrast with other well-known regulators of food intake

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and the reproductive axis, such as leptin, NPY, orexin, GALP, and likely ghrelin, for which direct, independent actions in the control of feeding and reproductive axis have been reported (9, 29, 32, 33), and strengthens the role of the KiSS-1 system as a selective downstream regulatory signal essential for the proper function of the GnRH-LH axis.

Additional evidence for the pivotal involvement of the KiSS-1 system in the mechanisms whereby energy status controls activation of the reproductive axis at puberty is provided by our experiments involving chronic administration of kisspeptin in conditions of undernutrition. In our setting, a persistent decrease in daily food intake of 30% from controls was able to totally block normal pubertal development, as estimated by lack of vaginal opening and decreased serum gonadotropin levels. In this model, repeated administration of kisspeptin was sufficient to restore vaginal opening in a significant number (60%) of cases as well as to induce gonadotropin and estrogen secretion in every single animal tested. On the basis of present and previous data (18, 19, 23), this activational response is thought to be mediated by central stimulation of the GnRH system. Yet, in keeping with our previous results (24), such an effect did not involve increased hypothalamic expression of GnRH mRNA, nor was it associated with significant changes in the relative levels of GnRH receptor mRNA at the pituitary (our unpublished observations). The reasons for the lack of vaginal opening in a proportion of food-restricted females injected with kisspeptin remain to be solved, but subtle individual variations in the responses to subnutrition and/or kisspeptin administration cannot be excluded. Moreover, it is plausible that occurrence of vaginal opening might have taken place in the remaining kisspeptin-injected animals (which actually had significantly elevated LH and estradiol levels over vehicle-treated underfed females at d 37) if longer treatment protocols could have been implemented.

The relevance of the present observations is stressed by the fact that, although kisspeptin was able to rescue vaginal opening in a large percentage of food-restricted females, the very same treatment protocol (between d 30 and 37 postpartum) dramatically depressed serum LH levels and prevented normal occurrence of canalization of the vagina in more than 50% of normally fed female rats at puberty (manuscript in preparation), *i.e.* when full activation of the endogenous KiSS-1 system occurs (17). This illustrates the exquisite sensitivity of this system not only to down-regulation (as observed in food deprivation) but also to hyperactivation, where desensitization of the gonadotropic axis is likely taking place. Additional studies, involving repeated administration of kisspeptin at different stages of maturation, and testing of hypothalamic secretion and pituitary sensitivity to GnRH, are presently in progress in our laboratory to cover this relevant issue. Nonetheless, although ultimate occurrence of ovulation as definitive proof of complete puberty was not monitored in our experimental setting, in the context of the reported decrease in KiSS-1 expression in situations of negative energy balance, our present data (involving assessment of different end-points such as pituitary LH and FSH secretion, ovarian-derived estrogen levels, and vaginal opening as biomarker of estrogenic action) strongly suggest that restoration of endogenous KiSS-1 tone is sufficient to rescue (at least partially) defective pubertal activation of the reproductive axis in undernutrition. This is not only relevant from a mechanistic standpoint, but it may pose also interesting therapeutic implications, especially considering the extraordinarily potent gonadotropin-releasing activity of systemically delivered kisspeptins (24, 25).

In summary, we provide herein an integral analysis of the potential interaction of energy balance and the KiSS-1 system in the control of reproductive function, with special attention to the role of this novel signaling system in conveying the impact of (insufficient) body energy stores onto the gonadotropic axis at puberty. Our data demonstrate for the first time that, despite its lack of direct effects on feeding and related neuropeptide gene expression, food deprivation induces significant changes in the expression and function of the central (hypothalamic) KiSS-1 system, which may represent a previously uncharacterized target for disruption (and eventual therapeutic intervention) of pubertal development in conditions of negative energy balance.

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