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Regulation of Hypothalamic Expression of KiSS-1 and GPR54 Genes by Metabolic Factors: Analyses Using Mouse Models and a Cell Line

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It is well established that reproductive function is metabolically gated. However, the mechanisms whereby energy stores and metabolic cues influence fertility are yet to be completely deciphered. Recently, the hypothalamic KiSS-1/GPR54 system has emerged as a fundamental regulator of the gonadotropic axis, which conveys the modulatory actions of sex steroids to GnRH neurons. Evidence is also mounting that KiSS-1 neurons may also represent the link between systemic metabolic signals and central control of reproduction. To further explore this possibility, we examined the impact of changes in energy status and key metabolic regulators on the hypothalamic expression of KiSS-1 and GPR54 genes, using different mouse models and the hypothalamic cell line N6. Time-course analysis of the effects of short-term fasting revealed a rapid (12- and 24-h) decline in KiSS-1 and GPR54 mRNA levels, which preceded that of GnRH (48 h). In contrast, diet-induced obesity or obesity associated with leptin deficiency (*ob/ob vs.* wild-type mice) failed to induce overt changes in hypothalamic expression of KiSS-1 and GPR54 genes. However, leptin infusion of *ob/ob* mice evoked a significant increase in KiSS-1 and GPR54 mRNA levels compared with pair-fed controls. Moreover, leptin, but not insulin or IGF-I, stimulated KiSS-1 mRNA expression in the mouse hypothalamic cell line N6. In addition, neuropeptide Y (NPY) null mice showed decreased KiSS-1 mRNA levels at the hypothalamus, whereas exposure to NPY increased expression of KiSS-1 in hypothalamic N6 cells. In sum, our present data further characterize the functional relevance and putative key mediators (such as leptin and NPY) of the metabolic regulation of the hypothalamic KiSS-1 system in the mouse. (*Endocrinology* 148: 4601–4611, 2007)

IT HAS LONG BEEN recognized that metabolic signals and the state of energy reserves of the organism are key modulators of reproductive function (1). Thus, full activation of the hypothalamic-pituitary-gonadal axis at puberty and its proper functioning in adulthood critically depend on sufficient body energy stores (1–3). Moreover, situations of persistent negative energy balance, such as extreme physical exercise and undernutrition, as well as disturbed metabolic conditions are often characterized by lack of puberty onset and reproductive failure (1). Although the ultimate signals and mechanisms responsible for the metabolic gating of reproductive function remain to be completely elucidated, our knowledge in this area has been significantly enlarged by the identification of the adipose hormone leptin, which signals the magnitude of energy stores to the hypothalamic centers governing reproduction (4, 5). Yet, a conundrum that has long persisted unsolved is that despite the pivotal role of leptin, GnRH neurons do not apparently express leptin receptors, suggesting the existence of intermediary regulatory mechanisms (6).

In the last years, the hypothalamic KiSS-1/GPR54 system has emerged as essential gatekeeper of GnRH neurons and, hence, of reproductive function. Interestingly, this ligand/receptor system was initially identified in the context of tumor biology (7). Thus, kisspeptins, the peptide products of the KiSS-1 gene that include kisspeptin-10 and metastin, were shown to act via the G protein-coupled receptor (GPR54) to suppress tumor metastasis (8–10). However, in late 2003, two independent reports disclosed the presence of inactivating mutations and deletions of the GPR54 gene in rare forms of hypogonadotropic hypogonadism (11, 12), a phenotype similar to that of GPR54 null mice (12, 13). Those findings initiated a series of molecular and pharmacological studies, mostly in laboratory rodents, aimed at the characterization of the physiological roles of kisspeptin and GPR54 in the control of reproduction (for a review, see Refs. 14 and 15). Some of the most salient reproductive features of this system, substantiated during the last 2 yr, can be summarized as follows: 1) kisspeptins are extraordinarily potent stimulators of the GnRH/gonadotropin axis (14, 16); 2) the KiSS-1 system plays an essential role in the timing of puberty onset (14, 17, 18); 3) the stimulatory actions of kisspeptins on the gonadotropic axis are primarily conducted at the hypothalamus, where they can activate GnRH neurons and elicit GnRH secretion (14, 16); and 4) hypothalamic expression of

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Abbreviations: DIO, Diet-induced obese; GNX, gonadectomized; GPR54, G protein-coupled receptor; HFD, high-fat diet; LFD, low-fat diet; LSD, least significant difference; NPY, neuropeptide Y; qrt, quantitative real-time; T, testosterone; wt, wild type.

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KiSS-1 gene is under the control of sex steroids, and KiSS-1 neurons are involved in mediating the negative and positive feedback effects of estradiol on gonadotropin secretion (14, 19). Moreover, the hypothalamic KiSS-1 system is likely to operate also as a molecular conduit for the control of the gonadotropic axis by additional relevant regulators, such as environmental cues and metabolic signals (15, 20).

Concerning its potential involvement in the metabolic control of fertility, initial studies in pubertal rats demonstrated suppressed hypothalamic expression of the KiSS-1 gene in situations of negative energy balance associated with decreased gonadotropin secretion (21). More recently, impairment of hypothalamic KiSS-1 expression was also observed in adult male rats with altered metabolic conditions linked to hypogonadotropic hypogonadism, such as uncontrolled experimental diabetes (22). Interestingly, in both models, acute or repeated administration of kisspeptin efficiently rescued the state of hypogonadotropism (21, 22). Moreover, studies in diabetic rats suggested that leptin, whose circulating levels significantly decrease in this model, plays a stimulatory role on hypothalamic KiSS-1 expression, thus serving an important function in the metabolic control of this system. This contention has been independently supported by data from orchidectomized *ob/ob* mice, which showed decreased expression of KiSS-1 mRNA in the arcuate nucleus that was partially restored by leptin administration (23).

Although the above data collectively point to a major role of the hypothalamic KiSS-1/GPR54 system in conveying the regulatory effects of energy balance and metabolic cues on the gonadotropic axis, several aspects of this critical function remain poorly defined. For instance, the time course for the dampening of hypothalamic KiSS-1 mRNA levels after food deprivation as well as the potential impact of extreme obesity on this system have not been yet characterized. Likewise, our knowledge of the signals involved in the metabolic control of KiSS-1 and their putative sites of action remains limited. To address these issues, we report herein a comprehensive series of studies on the expression levels of KiSS-1 and GPR54 at the hypothalamus of relevant mouse models [short-term fasting, diet-induced obesity, *ob/ob*, and neuropeptide Y (NPY) knockout]. In addition, expression of KiSS-1 and GPR54 genes was also evaluated in the murine hypothalamic cell line N6 (24) after challenge with well-known regulators of food intake, energy balance, and reproduction, such as leptin, NPY, insulin, and IGF-I (5, 24–27).

Materials and Methods

Animals and cell culture

All experimental procedures were approved by the Animal Care and Use Committees of the University of Illinois at Chicago and the Jesse Brown Veterans Affairs Medical Center. C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) at 7 wk of age and allowed to acclimate to the facility, personnel, and daily handling. During this acclimation period, mice were housed under standard conditions of light (12-h light, 12-h dark cycle, lights on at 0700 h) and temperature (22–24 C), with free access to standard rodent chow (LabDiet, catalog no. 5008: fat, 17 kcal%; carbohydrate, 56 kcal%; protein, 27 kcal%) and tap water unless otherwise specified. All mice were killed at adult age by decapitation without anesthesia between 0700–0900 h. Immediately upon decapitation, hypothalami and pituitaries were collected and frozen in liquid nitrogen and stored at −80 C until analysis of hypothalamic KiSS-1, GPR54, and GnRH and pituitary LHβ mRNA levels by quantitative real-time (qrt)RT-PCR (see below for details). Descriptions of the experiments conducted follows.

Effect of fasting on the expression of KiSS-1/ GPR54 system. C57BL/6J male mice at 10 wk of age were assigned to one of four groups (n = 5–8): 12-h fasting (food removed at 1900 h), 24-h fasting, 48-h fasting (food removed at 0700 h), or feeding *ad libitum*, as previously reported (28).

Expression of KiSS-1/GPR54 system in diet-induced obesity. To evaluate the effect of the obese state on hypothalamic expression of KiSS-1 and GPR54 genes, tissue samples were obtained from diet-induced obese (DIO) male mice, fed a low-fat diet (LFD) or a high-fat diet (HFD) diet as previously reported (29). Briefly, groups of C57BL/6J male mice (n = 6–7) were placed on either a LFD (10% kcal from fat, used as control) or HFD (60% kcal from fat) at 4 wk and killed at 20 wk of age.

Effects of leptin on expression of KiSS-1/ GPR54 system in ob/ob mice. To study the impact of leptin replacement on the hypothalamic expression of KiSS-1 and GPR54 genes in the *ob/ob* mouse, tissue samples from *ob/ob* male mice (10 wk of age) used in a recent study (30) were further evaluated. Briefly, groups of *ob/ob* mice (n = 5) were anesthetized with ketamine/xylazine and implanted sc with osmotic mini-pumps containing either recombinant mouse leptin or vehicle for 7 d, resulting in circulating leptin levels that were similar to wild-type (wt) controls (~1 ng/ml). This study included a group of *ob/ob* mice pair-fed to match the food intake of leptin-treated mice, to differentiate between direct effects of leptin and those mediated indirectly by leptin-induced reduction in food intake and weight loss.

Expression of KiSS-1/GPR54 system in NPY null mice. To explore the potential role of endogenous NPY in the regulation of KiSS-1 and GPR54 expression, hypothalamic samples from male NPY-knockout ($NPY^{-/-}$) and NPY-intact ($NPY^{+/+}$) mice of the 129/sv strain were used, as previously reported (28, 31). Briefly, $NPY^{-/-}$ mice were provided by Dr. Richard D. Palmiter (Howard Hughes Medical Institute, University of Washington, Seattle, WA) (32) and maintained as a homozygous colony on a 129/sv background. Male $NPY^{+/+}$ mice (129/sv) were purchased from Jackson Laboratory. Mice were fed *ad libitum* or subjected to food deprivation for 48 h. After this period, groups of mice (n = 5–6 per group; 9–11 wk old) were killed by decapitation and tissues collected for analysis. Of note, hypothalami from all experimental groups ($NPY^{-/-}$ and $NPY^{+/+}$, either fed *ad libitum* or fasted) were processed and assayed simultaneously.

Besides KiSS-1 and GPR54, the expression levels of GnRH and LHβ mRNAs were evaluated in parallel, at the hypothalamus and pituitary, respectively, in mice from all experiments indicated above.

Effects of leptin, NPY, insulin, and IGF-I on KiSS-1 and GPR54 mRNA levels in the mouse hypothalamic cell line N6. To investigate whether expression of KiSS-1 and GPR54 genes as well as that of GnRH could be directly regulated by metabolic hormones in hypothalamic cells, we used the mouse hypothalamic cell line N6 (CELLutions Biosystems Inc., Ontario, Canada), originally developed by Belsham *et al.* (24); a detailed expression profile is provided at www.cellutionsbiosystems.com/n6.php. This cell line was maintained according to previously described methods (24). N6 cells were cultured in monolayer at 1×10^6 cells per well in α-MEM (Invitrogen Life Technologies, Inc., Grand Island, NY) containing 2.5% fetal bovine serum (Sigma Chemical Co., St. Louis, MO), 0.1% BSA (Sigma), 6 mM HEPES (Invitrogen), transferrin (125 nM; Sigma), T_3 (0.6 nM; Sigma), hydrocortisone (275 nM; Sigma), and 1% penicillin-streptomycin antibiotic (Invitrogen) and maintained at 37 C in an atmosphere of 5% CO_2. After 24 h incubation, cultures were preincubated in serum-free media for 2 h, and subsequently the medium was replaced with medium alone or containing 1 ng/ml leptin, 10 nM NPY, insulin, or IGF-I (three to four wells per treatment group). Cultures were incubated for an additional 24 h and total RNA recovered (see below).

RNA isolation and RT

Tissues and cell cultures were processed for recovery of total RNA using the Absolutely RNA RT-PCR Miniprep Kit (Stratagene, La Jolla, CA), with DNase treatment as previously described (29, 30). The amount of RNA recovered was determined using the Ribogreen RNA quantification kit (Molecular Probes, Eugene, OR). Total RNA (1 μg) of whole tissue extracts or cell culture extracts was reverse transcribed in a 20-μl

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volume using random hexamer primers, with enzyme and buffers supplied in the cDNA First Strand Synthesis kit (MRI Fermentas, Hanover, MD). cDNA was treated with RNaseH (MRI Fermentas), and duplicate aliquots (1 μl) were amplified by qrtRT-PCR, where samples were run against synthetic standards to estimate mRNA copy number (see below).

Primer selection, development, and validation of qrtRT-PCR

Details regarding development, validation, and application of a qrtRT-PCR to measure expression levels of mouse transcripts have been previously reported by our laboratory (28–30). Primers used for real-time RT-PCR were selected using mouse genomic sequences as templates and primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/ primer3 www.cgi; Steve Rozen, Whitehead Institute for Biomedical Research, Cambridge, MA). Primer sets for mouse KiSS-1, GPR54, GnRH, and LHβ used in this study have not been previously reported and therefore are provided in Table 1. This table also contains descriptions of the primer pairs used for amplification of additional relevant targets in N6 cells. Sequences of selected primers were used in BLAST (NCBI) searches to check for potential homology to sequences other than the designated target. For real-time PCR, Brillant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA) was used, where thermocycling and fluorescence detection was performed using a Stratagene Mx3000p real-time PCR machine. The final volume of the PCR was 25 μl: 1 μl RT sample, 12.5 μl QPCR Master Mix, 0.375 μl of each primer (10 μM stock solution), 0.375 μl reference dye, and 10.375 μl dH_2O. The thermal cycling profile consisted of a preincubation step at 95 C for 10 min, followed by 40 cycles of denaturation (95 C, 30 sec), annealing (61 C, 1 min), and extension (72 C, 30 sec). Final PCR products were subjected to graded temperature-dependent dissociation to verify that only one product was amplified. To determine the starting copy number of cDNA, RT samples were PCR amplified and the signal compared with that of a standard curve run on the same plate. Standard curves consisted of 10^1, 10^2, 10^3, 10^4, 10^5, and 10^6 copies of synthetic cDNA template for each of the transcripts of interest. Standard curves were generated by the Stratagene Mx3000p Software, and the slopes were approximately 1, indicating the efficiency of amplification was 100%, meaning that within the detectable range, all templates in each cycle were copied. In addition, total RNA samples that were not reverse transcribed and a no-DNA control were run on each plate to control for genomic DNA contamination and exogenous contamination, respectively. Also, to control for variations in the amount of RNA used in the RT reaction and the efficiency of the RT reaction, mRNA copy number of the transcript of interest was adjusted by the mRNA copy number of cyclophilin A (a peptidyl isomerase), where cyclophilin A mRNA levels did not significantly vary between experimental groups, within tissue type (data not shown). Details regarding the development, validation, and application of a qrtRT-PCR to measure expression levels of mouse cyclophilin A have been previously reported (29).

Hormone assays

Changes in serum leptin levels were monitored in selected experimental groups (time-course fasting and DIO mice fed on HFD and LFD), using a leptin ELISA kit (mouse ELISA; Linco Research Inc., St. Charles, MO) following the instructions of the manufacturer. In addition, serum testosterone (T) levels were measured in all the experimental groups using an ELISA kit from DRG International (Mountainside, NJ) following the procedure provided by the supplier.

TABLE 1. Mouse-specific primers for amplification of transcripts of KiSS-1, GPR54, GnRH, and LHβ genes as well as genes encoding NPY, leptin, insulin, and IGF-I receptors (NPY-Y1 to Y6, leptin-R, insulin-R, IGFI-R), used for qrtRT-PCR

Gene	GenBank accession no.	Primer sequence	Nucleotide position	Product size (bp)
Kiss-1	AB162440			127
Sense		AGCTGCTGCTTCTCCTCTGT	Sn 23	
Antisense		GCATACCGCGATTCCTTTT	As 149	
GPR54	AC151846			186
Sense		GCCACAGACGTCACTTTCCTAC	Sn 256	
Antisense		CGGGAACACAGTCACATACCA	As 441	
GnRH	NM_008145			144
Sense		GCATTCTACTGCTGACTGTGTGTT	Sn 23	
Antisense		GTTCTGCCATTTGATCCACCT	As 166	
LHβ	Y10418			138
Sense		TGTCCTAGCATGGTCCGAGT	Sn 123	
Antisense		AGGAAAGGAGACTATGGGGTCTA	As 260	
NPY-Y1	BC051420			101
Sense		ATGATCTCCACCTGCGTCAA	Sn 1066	
Antisense		CGAGACCGGAAGTCACAAAA	As 1166	
NPY-Y2	D86238			125
Sense		GTGTGCGTGGTAGTGGTGTTT	Sn 1099	
Antisense		ATGTGGAACACGGTGAAGATG	As 1223	
NPY-Y4	AK035505			200
Sense		AGGTCGTCTGCTTTGTGTCCT	Sn 883	
Antisense		GTTGATCCGCTTCATCTGTCC	As 1082	
NPY-Y5	AF049329			228
Sense		GAGAAGCACCTAACCGTTCCA	Sn 989	
Antisense		CATCCAGCTAACAGCGAACAC	As 1216	
NPY-Y6	BC103620			
Sense		TGATGGACCACTGGGTATTTG	Sn 531	
Antisense		GGCATGAGCTACTCTGGGTTT	As 691	161
Leptin-R	U42467			
Sense		GGAAGGAGTTGGAAAACCAAAG	Sn 2502	
Antisense		TCCGAGCAGTAGGACACAAGA	As 2628	127
Ins-R	NM_010568			
Sense		TCATGGATGGAGGCTATCTGG	Sn 3764	
Antisense		CCTTGAGCAGGTTGACGATTT	As 3892	129
IGF-IR	NM_010513			
Sense		TGGAGTGCTGTATGCTTCTGTG	Sn 2910	
Antisense		CTGGTTTCGGGTTCATCCTT	As 3089	180

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Data analysis

Samples from all groups within an experiment were processed at the same time, and therefore the *in vivo* effects of gender, genotype, and diet and the *in vitro* effects of leptin, NPY, insulin, and IGF-I on mRNA levels were assessed by Student's *t* test (*vs.* corresponding controls). The effects of time of fasting in C57BL6/SJ mice and leptin replacement in *ob/ob* mice were assessed by one-way ANOVA followed by Fisher's least significant difference (LSD) test for multiple comparisons. The effects of genotype and fasting on mRNA levels in NPY wt *vs.* NPY knockout mice were assessed by two-way ANOVA, followed by Fisher's LSD test. $P < 0.05$ was considered significant. All values are expressed as mean ± SEM. All statistical analyses were performed using the GB-STAT software package (Dynamic Microsystems, Inc., Silver Spring, MD).

Results

Time course of fasting effects on hypothalamic KiSS-1 and GPR54 gene expression

The impact of conditions of negative energy balance on the hypothalamic expression of the elements of the KiSS-1/GPR54 system, and its time-course profile, were explored in the mouse after different periods of fasting. Thus, expression levels of KiSS-1 and GPR54 mRNAs were assayed at the hypothalamus of C57BL/6J mice at different intervals after food deprivation (12, 24, and 48 h) and compared with the mRNA levels of GnRH at the hypothalamus and LHβ at the pituitary. In addition, serum T levels were also assayed in the experimental groups. Fasting evoked a significant reduction in body weight (9.6 ± 0.8% at 12 h, 11 ± 0.5% at 24 h, and 17 ± 1.1% at 48 h *vs.* controls fed *ad libitum*), which was associated with a significant lowering of serum leptin levels (0.11 ± 0.008 ng/ml at 12 h, 0.08 ± 0.03 ng/ml at 24 h, and 0.03 ± 0.01 ng/ml at 48 h *vs.* 0.27 ± 0.04 ng/ml in controls). Similarly, serum T levels were significantly decreased from 12 h fasting onward, T concentrations in food-deprived animals being less than 10% of those of fed controls (Fig. 1). As for leptin and T levels, fasting induced a significant decrease in hypothalamic expression of KiSS-1 mRNA, which was evident already at 12 h after food deprivation and was persistently detected up to 48 h of fasting. A similar profile of response to fasting was observed for hypothalamic GPR54 mRNA. In contrast, relative levels of GnRH mRNA remained invariant at 12 and 24 h after food deprivation, whereas a significant drop in its mRNA levels was detected after 48 h fasting. Finally, expression levels of LHβ mRNA at the pituitary remained unaltered during food deprivation at all time points studied (Fig. 1).

Effects of diet-induced obesity on hypothalamic KiSS-1 and GPR54 gene expression

The potential consequences of a state of persistent obesity on the hypothalamic expression of KiSS-1 and GPR54 genes were explored in a model of diet-induced obesity. In keeping with previous reports, DIO mice fed on a HFD for 16 wk became markedly overweight (44.7 ± 1.7 g body weight) and hyperleptinemic (2.53 ± 0.3 ng/ml), compared with mice fed on a LFD, used as reference controls (34.2 ± 0.6 g body weight; 1.25 ± 0.1 ng/ml). In this setting, no significant differences were observed in the expression levels of KiSS-1 and GPR54 mRNAs between HFD and LFD groups. Similarly, relative levels of GnRH and LHβ mRNAs were not significantly different between obese and lean animals. Strikingly, however, HFD mice were severely hypoandrogenic, with serum T values that were less than 10% of those of LFD animals (Fig. 2).

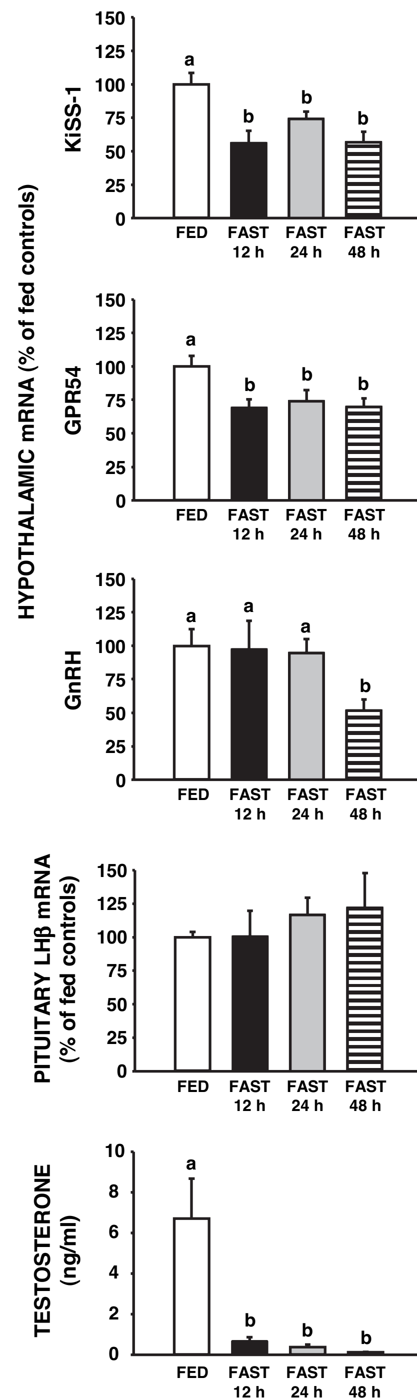

FIG. 1. Time course for the effects of fasting on the expression levels of KiSS-1, GPR54, and GnRH mRNAs at the hypothalamus as well as on LHβ mRNA at the pituitary and serum T concentrations in adult male mice. The animals were subjected to food deprivation (FAST) for different time intervals: 12, 24, and 48 h. Mice fed *ad libitum* served as controls (FED). Expression levels of the targets were obtained by qrtRT-PCR as described in *Materials and Methods*. Groups with different *superscript letters* are statistically different (one-way ANOVA followed by Fisher's LSD test).

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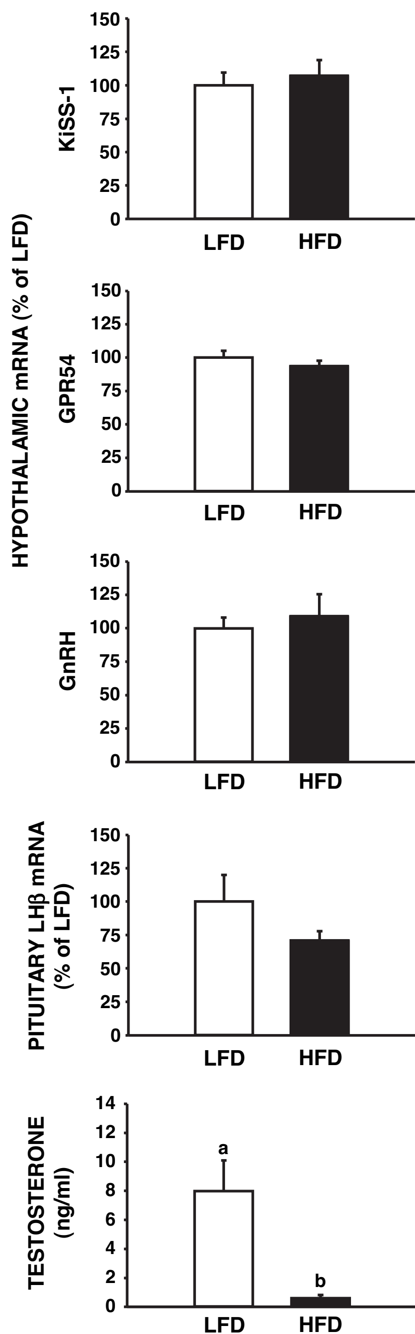

FIG. 2. Effect of diet-induced obesity on the expression levels of KiSS-1, GPR54, and GnRH mRNAs at the hypothalamus as well as on LHβ mRNA at the pituitary and serum T concentrations in the mouse. DIO male mice were fed a HFD for 16 wk to induce overweight; mice fed a LFD served as controls. Expression levels of the targets were obtained by qrtRT-PCR as described in *Materials and Methods*. For the different parameters under analysis, no significant differences were detected, except for testosterone concentrations, where groups with *different superscript letters* are statistically different (Student's *t* test).

Leptin replacement and hypothalamic KiSS-1 and GPR54 gene expression in ob/ob mice

The effects of leptin on the hypothalamic expression of the elements of the KiSS-1/GPR54 system were analyzed by means of continuous infusion of the hormone in *ob/ob* mice. In keeping with previous observations (21), relative levels of KiSS-1 and GPR54 mRNAs in whole hypothalamic preparations were not significantly different between wt and *ob/ob* mice; neither was there a change in hypothalamic GnRH mRNA levels. However, relative expression of LHβ mRNA at the pituitary was significantly decreased in leptin-deficient mice (data not shown), as were serum T levels (0.46 $\pm$ 0.05 *vs.* 6.8 $\pm$ 1.8 ng/ml in wt mice). Leptin infusion to *ob/ob* mice evoked a marked reduction in mean daily food intake over the study period (1.6 $\pm$ 0.1 g chow/d *vs.* 6.2 $\pm$ 0.5 g in *ob/ob* mice fed *ad libitum* on d 7 of treatment). Accordingly, an additional group of *ob/ob* mice were pair fed to match the food intake of animals infused with leptin to discriminate between genuine leptin effects and those caused by the decreased food intake. Indeed, such a procedure resulted in a net body weight loss of 14.8 $\pm$ 1.5%, which was roughly similar to that induced by leptin treatment in *ob/ob* animals (16.5 $\pm$ 1.7%). This protocol of food restriction caused a significant reduction in hypothalamic levels of KiSS-1 and GPR54 mRNAs in *ob/ob* mice but did not affect the relative mRNA levels of GnRH and LHβ, consistent with short-term effects (12 and 24 h) of complete food withdrawal in wt mice. In addition, food restriction evoked a significant reduction in serum T levels in *ob/ob* mice (Fig. 3). On the other hand, although leptin administration did not alter KiSS-1/GPR54 levels in *ob/ob* mice fed *ad libitum*, infusion of leptin did evoke a significant elevation of KiSS-1 and GPR54 mRNA levels when food intake was restricted. Of note, leptin also caused a marked rise in the expression of GnRH and LHβ mRNAs as well as in circulating T levels, which clearly exceeded those detected in untreated *ob/ob* mice (Fig. 3).

Hypothalamic KiSS-1 and GPR54 expression in fed and fasted NPY null mice

Given the prominent roles of NPY in the metabolic regulation of the reproductive axis (25) and the proven interaction between leptin and NPY signaling (5, 25), we sought to determine the potential function of NPY in the control of the elements of the KiSS-1/GPR54 system. To this end, their hypothalamic expression was analyzed, in basal conditions and after 48 h fasting, in NPY knockout mice. Although caution should be exercised in the direct comparison of the wt and NPY knockout strains in that the NPY knockouts were maintained as an inbred homozygous colony and thus could have diverged over time from the original 129sv background, it was interesting to note that lack of NPY gene resulted in a significant decrease of KiSS-1 and, to a lesser extent GPR54, mRNA copy numbers at the hypothalamus, although the latter did not reach statistical significance (Fig. 4). Also of note, GnRH mRNA levels were not significantly different between wt and NPY null mice, but the latter showed a reduction in LHβ mRNA content at the pituitary ($P < 0.05$ *vs.* corresponding wt controls after Student's *t* test). Likewise, a trend toward reduction in serum T levels, which did not reach statistical significance, was observed in NPY null mice (Fig. 4).

In wt 129/sv mice, food deprivation for 48 h induced a significant decrease in hypothalamic KiSS-1 and GPR54

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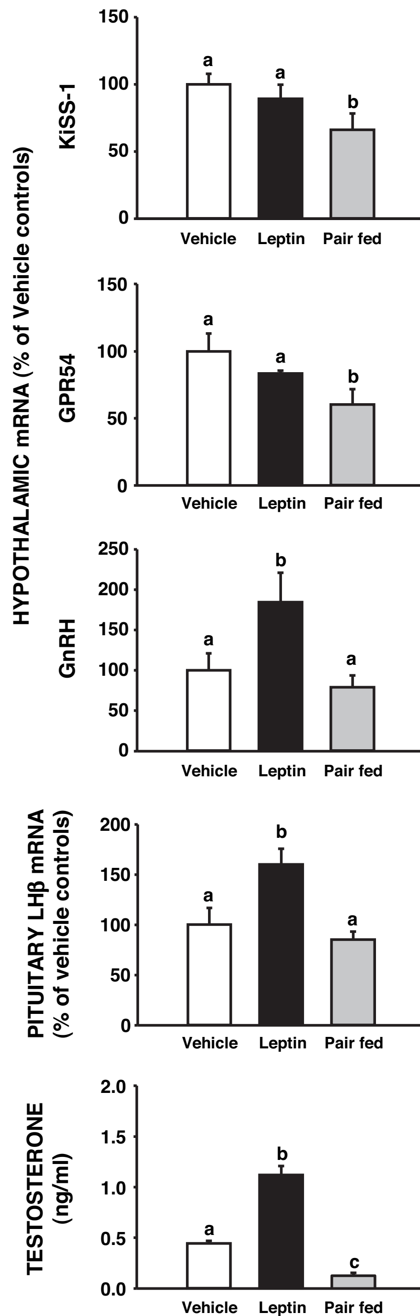

FIG. 3. Effect of leptin infusion on the expression levels of KiSS-1, GPR54, and GnRH mRNAs at the hypothalamus as well as on LHβ mRNA at the pituitary and serum T concentrations in adult *ob/ob* mice. Vehicle-infused *ob/ob* animals served as controls. In addition, because leptin treatment evoked a significant reduction in daily food intake, a group of *ob/ob* mice infused with vehicle and pair-fed along with leptin-treated animals was used as (additional) control. Expression levels of the targets were obtained by qrtRT-PCR as described in *Materials and Methods*. Groups with different *superscript letters* are statistically different (one-way ANOVA followed by Fisher's LSD test).

mRNAs, which was associated with the lowering of GnRH (but not of LHβ) mRNA levels and drop in serum T concentrations, in keeping with data obtained in C57BL/6J mice. In NPY knockout mice, 48 h fasting evoked gene expression responses that were grossly similar to those observed in wt

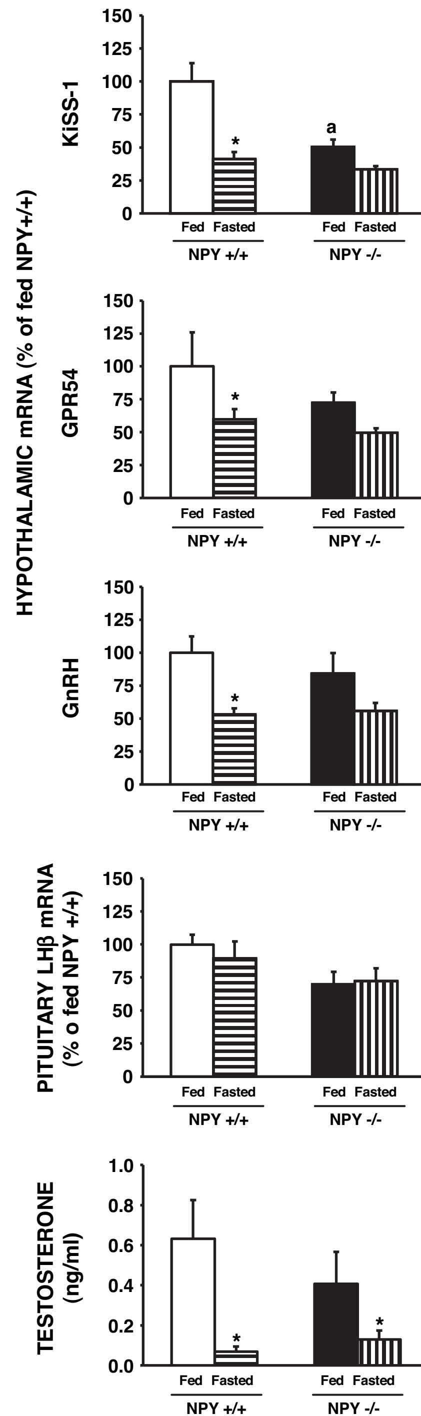

FIG. 4. Expression levels of KiSS-1, GPR54, and GnRH mRNAs at the hypothalamus as well as of LHβ mRNA at the pituitary and serum T concentrations in NPY null (−/−) mice. The wt (+/+) animals served as controls. In addition to basal levels, the effect of 48 h fasting on the expression of those mRNAs was explored in wt and NPY knockout animals. Expression levels of the targets were obtained by qrtRT-PCR as described in *Materials and Methods*. *, $P < 0.05$ *vs.* corresponding groups fed *ad libitum*; a, $P < 0.05$ *vs.* corresponding NPY +/+ groups (two-way ANOVA followed by Fisher's LSD test). Of note, direct comparison of LHβ mRNA levels at the pituitary in NPY +/+ and −/− mice revealed a statistically significant decrease in knockout animals ($P < 0.05$; Student's *t* test).

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animals (decrease in KiSS-1, GPR54, and GnRH mRNA levels). Yet, due to the prevailing lowering of expression levels (KiSS-1) and/or sample variability among the experimental groups (GnRH), such differences were below the limit of statistical significance. In addition, serum T levels were significantly decreased by fasting in NPY null mice (Fig. 4).

KiSS-1, GPR54, and GnRH mRNA expression in the hypothalamic cell line N6: effects of leptin, insulin, IGF-I, and NPY

Finally, to address direct regulatory actions of key metabolic signals, with proven roles in the control of energy balance, food intake, and reproductive function, we examined the effects of leptin, insulin, IGF-I, and NPY on KiSS-1 and GPR54 mRNA levels in the mouse hypothalamic cell line N6. This cell line was originally developed by Belsham and co-workers (24), as described in detail elsewhere; an overall expression profile of this cell line can be obtained at www.cellutionsbiosystems.com/n6.php. For the purposes of this study, we initially analyzed the absolute expression levels in this cell line (expressed as cDNA copy number/0.05 μg total RNA) of the following mRNA targets: KiSS-1, GPR54, GnRH, NPY receptor 1 (-Y1), NPY-Y2, NPY-Y4, NPY-Y5, NPY-Y6, leptin receptor, insulin receptor, and IGF-I receptor; levels which were compared with those of the same targets in mouse hypothalamus. Primer sets for mouse NPY-Y1, NPY-Y2, NPY-Y4, NPY-Y5, NPY-Y6, leptin receptor, insulin receptor, and IGF-I receptor used in this study have not been previously reported and therefore are also provided in Table 1. As shown in Table 2, N6 cells express KiSS-1 and GPR54 mRNAs as well as that of GnRH. The level of expression of these transcripts in the N6 cell line was about 50% of those observed in whole hypothalamic extracts except for GnRH, which was about 16%. In addition, the mRNAs encoding different NPY-receptor subtypes (from Y1 to Y6) were detected in the N6 cell line. Yet, their absolute levels of expression considerably varied among the different subtypes (with moderate to negligible expression for NPY-Y1, NPY-Y2, and NPY-Y5 but high expression levels for NPY-Y4 and NPY-Y6 mRNAs). Moreover, when compared with absolute expression levels in whole hypothalamic preparations, it became evident that whereas NPY-Y1, NPY-Y2, and NPY-Y5 are expressed at relatively low abundance in N6 cells (4, 1, and 8.5% of total hypothalamus, respectively), NPY-Y4 and NPY-Y6 mRNAs were significantly enriched in this neuronal cell line (with relatives expressions of 163 and 121% of total hypothalamus, respectively). In addition, the mRNAs encoding the receptors for leptin, insulin, and IGF-I were highly expressed in N6 cells at levels that equaled or exceeded those in whole hypothalamic tissue.

TABLE 2. Absolute cDNA copy number/0.05 μg total RNA of gene transcripts in the hypothalamus of C57BL/6 mice *vs.* the hypothalamic N6 cell line, as determined by qrtRT-PCR

	cDNA copy number/0.05 μg total RNA	
	Mouse hypothalamus	Hypothalamic N6 cell line
Cyclophilin	441,586 ± 64,850	466,805 ± 84,165
KiSS-1	503 ± 54	273 ± 48
GPR54	4003 ± 433	1725 ± 379
GnRH	765 ± 78	117 ± 12
NPY-Y1	3767 ± 178	145 ± 0.4
NPY-Y2	4372 ± 656	44 ± 0.4
NPY-Y4	299 ± 15	490 ± 180
NPY-Y5	1585 ± 13	135 ± 19
NPY-Y6	587 ± 63	707 ± 39
Leptin-R	184 ± 39	200 ± 22
Ins-R	760 ± 95	837 ± 138
IGF-IR	2571 ± 577	3629 ± 324

Values are means ± SEM (n = 3–39 tissues or wells per group).

Given that N6 cells expressed KiSS-1, GPR54, and GnRH and receptors for multiple metabolic signals, we next used this cell line as a model to examine the direct effects of leptin, insulin, IGF-I, and NPY on the KiSS-1 system. As shown in Fig. 5A, a 24-h challenge with leptin (1 ng/ml) evoked a significant elevation of KiSS-1 and GnRH mRNAs in N6 cells. In contrast, the relative levels of GPR54 mRNA were not significantly altered after leptin stimulation. Insulin and IGF-I (10 nM) did not alter KiSS-1 and GPR54 expression but did clearly stimulate the expression of GnRH (Fig. 5B). Finally, challenge of N6 cells with 10 nM NPY induced a significant increase in KiSS-1 mRNA levels, which was not associated with changes in the expression of GPR54 or GnRH mRNAs (Fig. 5C). For all the experiments described above, the doses of the different hormonal stimuli were selected on the basis of previous publications, demonstrating their effectiveness in equivalent *in vitro* models (33, 34).

Discussion

We report herein a comprehensive series of analyses, using different *in vivo* models and the hypothalamic N6 cell line, which aim to explore the role of energy status and key metabolic regulators in the control of the hypothalamic KiSS-1/GPR54 system in the mouse. Of note, although it is generally assumed that the female is more sensitive to metabolic regulation of fertility (1), our *in vivo* studies were conducted in male mice. This procedure allowed us to exclude the potential bias of cyclic fluctuations of reproductive function in adult females. More importantly, male rodents are also subjected to the modulation of reproductive function by energy status and metabolic signals (1), and hypothalamic expression of KiSS-1 gene in the male mouse is mostly confined to the arcuate nucleus (14, 16, 19), which made it especially suitable for our expression analyses using qrtRT-PCR. Admittedly, however, our analytical approach does not interrogate other levels of potential regulation of this system, such us changes in protein expression or activity of kisspeptin and GPR54. Moreover, although improbable in male rodents, our procedure cannot rule out the possibility of divergent, compensatory changes in KiSS-1 or GPR54 mRNA levels between different hypothalamic nuclei.

The present study confirms our previous observations in the rat showing that states of negative energy balance are linked to suppressed expression of KiSS-1 gene at the hypothalamus (21) and defines for the first time the temporal course for the effects of fasting on KiSS-1 mRNA levels. Indeed, although the possibility of an inhibition of hypothalamic KiSS-1 system in conditions of energy insufficiency (which are invariantly associated with defective gonadotropic function) was mechanistically appealing, to our knowledge, the studies so far reported on this aspect of

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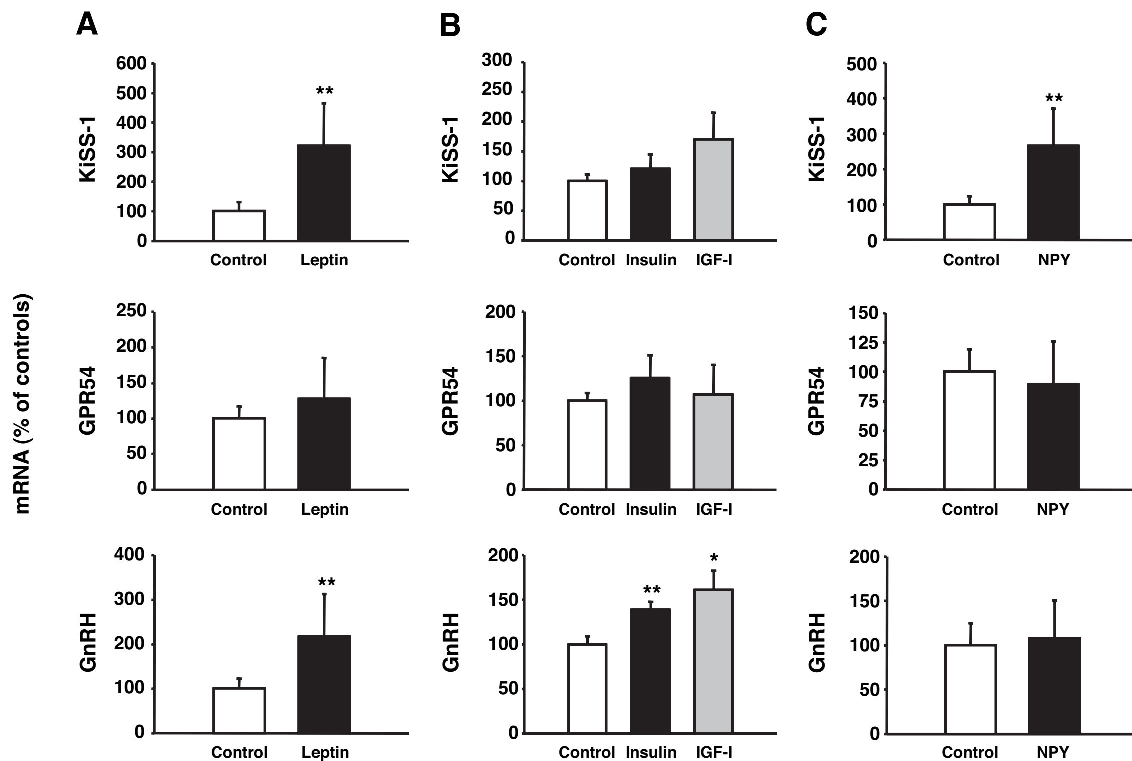

FIG. 5. Expression levels of KiSS-1, GPR54, and GnRH mRNAs in the murine hypothalamic cell line N6. Cells were challenged with effective doses of leptin (A), insulin (B), IGF-I (B), or NPY (C) and harvested after 24 h for RNA analysis. Expression levels of the targets were obtained by qrtRT-PCR as described in *Materials and Methods*. *, $P < 0.05$; **, $P < 0.01$ *vs.* corresponding controls (one-way ANOVA followed by Fisher's LSD test).

KiSS-1 physiology had been restricted to one species (rat) and mostly after longer periods of either food deprivation (>60 h) or metabolic disturbance (21, 22). In our present study, a rapid decline (<12 h) in the hypothalamic expression of KiSS-1 transcript was detected upon food deprivation, with persistently decreased mRNA levels along the 48-h period of fasting. This profile of expression closely paralleled that of circulating leptin after short-term fasting, with a precipitous decrease of leptin concentrations as soon as 12 h after food deprivation, a fall that might operate as a causative factor for the decline in KiSS-1 mRNA levels (22, 23) (present results). Interestingly, GPR54 mRNA levels mirrored the changes detected for KiSS-1 transcript along the period of 48 h fasting. Yet, in the rat, a moderate but significant increase in GPR54 expression had been previously reported by our group after 60 h fasting (21). Besides potential species differences, one possible explanation for this discordant observation is that food deprivation might initially evoke a decline in GPR54 gene expression that would be overcome in the mid to long term (>48 h) by a sensitization mechanism, involving increased GPR54 mRNA expression due to a persistent decrease in the endogenous tone of the ligand (21). Finally, it is also notable that GnRH mRNA levels declined only after 48 h fasting, *i.e.* much later than the suppression of KiSS-1 gene expression became detectable. This observation is grossly coincident with previous data showing that GnRH gene expression itself is relatively insensitive to the effects of food deprivation in the rat (35) and strongly suggests that the KiSS-1/GPR54 system is indeed a sensitive and rapid transducer of the state of body energy stores in rodents.

At the other extreme of the spectrum of energy unbalance, our present analyses failed to detect any significant effect of persistent overweight (and hyperleptinemia) on the hypothalamic expression of KiSS-1 and GPR54 genes. Notably, morbid obesity has been linked to variable degrees of hypogonadism (36), whose underlying mechanisms remains to be fully elucidated. Nonetheless, among the potential causative factors, a direct inhibitory effect of leptin on testicular steroidogenesis has been proposed in rodents and humans (36–39). This might explain, at least partially, the decrease in serum T levels observed in HFD mice (Fig. 2). In this context, our data seems to be consistent with a lack of primary negative effects of hyperleptinemia on gene expression of the hypothalamic (KiSS-1, GPR54, GnRH) and pituitary (LHβ) components of the gonadotropic axis. Yet, the fact that relative levels of KiSS-1 (and GPR54) mRNA were not increased in HFD mice, despite the severe reduction in serum T levels observed in this model, is worthy of consideration. Indeed, gonadal steroids have been proven as key regulators of hypothalamic KiSS-1 gene expression, and KiSS-1 mRNA levels have been reported to markedly increase in whole hypothalamic extracts after gonadectomy (39). Thus, it is tempting to hypothesize that expression of KiSS-1 gene in this model of obesity is actually inappropriately low, considering the concurrence of two potential stimuli in HFD mice: 1) decreased T levels and 2) increased circulating leptin (17) (present re-

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sults). Altogether, these observations suggest that, besides direct testicular actions (36–39), defects in the central mechanisms controlling the gonadotropic axis might also be taking place in this model of obesity and hyperleptinemia; a state of resistance to KiSS-1 up-regulation whose basis and functional relevance for the observed hypoandrogenism in DIO mice are yet to be defined. In addition, it is also possible that 1) changes in protein expression and/or activity of kisspeptin or GPR54 might take place in HFD mice, irrespective of variations of mRNA levels and/or 2) the period and magnitude of diet-induced obesity in our experiments might have not been great enough to evoke a full state of hypogonadotropic hypogonadism. These possibilities, which could not be directly assessed herein due to the lack of reliable semiquantitative protein assays for kisspeptin/GPR54 and the limited availability of serum samples from our models to measure LH and FSH (as direct index of gonadotropic function), warrant further investigation.

As initially reported by our group (21), intact *ob/ob* mice did not show significant changes in the relative levels of KiSS-1 and GPR54 mRNA in whole hypothalamic preparations. However, it should be noted that, as was the case for obese HFD animals, *ob/ob* mice are markedly hypogonadal, with serum T levels that were less than 10% of those of wt controls. Thus, in the *ob/ob* mouse, the putative inhibitory effects of the lack of leptin on KiSS-1 gene expression might be (in part) counteracted by the stimulatory effects of decreased sex steroid (T) levels. Therefore, it is reasonable to hypothesize that the conserved relative levels of KiSS-1 mRNA observed in the intact *ob/ob* mouse are disproportionately low for its expected expression in such a state of hypoandrogenism, suggesting the defective expression/function of this system in the absence of leptin actions. In fact, using gonadectomized (GNX) *ob/ob* males, Smith and colleagues (23) did recently demonstrate that KiSS-1 mRNA expression at the arcuate nucleus is actually lower in this model than in wt GNX mice.

Due to the above limitations, we concentrated our analyses on the effects of leptin replacement, by means of peripheral infusion of leptin for 7 d, on the expression levels of KiSS-1 and GPR54 genes at the hypothalamus. Considering that such a protocol of leptin administration induced a significant reduction in daily food intake, intact *ob/ob* mice pair-fed along with leptin-treated animals served as primary controls. In this setting, leptin replacement was able to evoke a significant increase in the hypothalamic expression levels of KiSS-1 and GPR54 mRNAs. Moreover, relative levels of GnRH and LHβ mRNAs as well as serum T concentrations were also elevated by leptin administration. Whereas these effects can be regarded as leptin-driven, it is intriguing to note that food restriction *per se* in leptin-deficient *ob/ob* mice induced a detectable suppression of hypothalamic KiSS-1 (and GPR54) mRNA levels (and circulating T), which suggests that, in this specific genetic background, at least part of the decrease in KiSS-1 expression linked to food deprivation might take place via leptin-independent mechanisms. Nonetheless, the ability of leptin to elicit KiSS-1 gene expression at the hypothalamus was further confirmed by our *in vitro* analyses using N6 cells, where challenge with an effective dose of leptin evoked a significant increase in KiSS-1 mRNA levels, which were associated with an elevation of GnRH mRNA expression. Whether the latter is a direct genuine action of leptin or is mediated via its effect on KiSS-1 expression remains to be elucidated. It is interesting to note, however, that using the very same setting, insulin and IGF-I were shown to increase GnRH mRNA levels in N6 cells, without affecting the relative levels of KiSS-1 and GPR54 transcripts. These observations are in good agreement with previous data showing the ability of insulin and IGF-I to increase GnRH expression in murine immortalized GnRH cells (26, 27, 40, 41) but the lack of effects of intracerebral infusion of insulin on hypothalamic KiSS-1 mRNA levels in the diabetic rat (22). Altogether, the above observations point out a major function of leptin in the metabolic control of the KiSS-1 system. Based on the comparison of data from models of leptin excess and deficiency reported herein, it is tempting to postulate that the physiological role of leptin is primarily related to the prevention of the fall of hypothalamic KiSS-1 mRNA expression observed in the undernourished state (permissive role) rather than with the net stimulation of KiSS-1 expression in conditions of energy abundance.

Notwithstanding, our observations in food-restricted *ob/ob* mice also suggest the possibility of additional metabolic regulators of KiSS-1 other than leptin. In this context, our study is the first to highlight the potential role of NPY in the regulation of hypothalamic expression of the KiSS-1 system, on the basis of two lines of evidence. First, KiSS-1 mRNA levels were consistently decreased in the hypothalamus of NPY null mice, which showed also a trend toward decrease in GPR54 mRNA expression (which did not reach statistical significance). Second, exposure to NPY *in vitro* elicited a significant elevation of KiSS-1 mRNA expression in the hypothalamic N6 cell line, without significantly affecting GPR54 or GnRH mRNA levels. Although, due to limited availability, replacement experiments in NPY null mice could not be conducted herein, the above findings jointly suggest that NPY is a positive regulator of KiSS-1 expression in the mouse. Of note, the actual role of NPY signaling in the central control of the gonadotropic axis has been the subject of intense debate, with stimulatory and inhibitory actions being reported depending on the pattern and time of administration and the subtype(s) of receptor recruited (42, 43). In any event, the fact that, despite reduced LHβ mRNA levels at the pituitary, NPY knockouts are not overtly infertile suggests that the lack of NPY does not cause a complete suppression of the hypothalamic KiSS-1 system. Moreover, the positive action of NPY reported herein is probably not related to the mechanism whereby leptin stimulates KiSS-1 gene expression, because leptin is known to repress NPY at specific hypothalamic (arcuate) circuitries (5, 44). Furthermore, NPY expression has been reported to increase after short-term fasting in mice (28), which suggest that changes in hypothalamic NPY are not likely to explain the rapid drop in KiSS-1 gene expression after food deprivation. Yet, our present data disclose the possibility that the proven stimulatory effects of NPY on some aspects of GnRH/LH secretion (42, 45) might be, at least partially, mediated by its actions on KiSS-1 expression.

In the present study, we took advantage of the immortalized hypothalamic cell line N6 for the analysis of direct

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effects of putative metabolic regulators of the hypothalamic KiSS-1 system. Detailed profiling demonstrated for the first time the expression of KiSS-1 and GPR54 genes of this cell line (see Table 2). In addition, the mRNAs encoding GnRH as well as the receptors for key metabolic regulators (such as leptin, insulin, IGF-I, and NPY) were also detected. Moreover, the fact that these ligands were able to evoke specific responses in terms of changes in KiSS-1 and/or GnRH expression demonstrates the functionality of such receptors in N6 cells. Altogether, these findings disclose the potential usefulness of this cell line for the analysis of the direct effects and putative mechanisms of action of relevant regulatory factors on the hypothalamic expression of KiSS-1 and GPR54 genes. Nonetheless, the physiological relevance of the above findings must be interpreted with caution, because N6 cells appear to jointly express KiSS-1, GPR54, and GnRH genes, whereas, at least in rodents, predominant expression of KiSS-1 and GPR54/GnRH seems to occur in distinct neuronal populations (KiSS-1 and GnRH neurons, respectively) (46, 47). Moreover, as might be the case with other immortalized cell lines used for the analysis of neuroendocrine mechanisms controlling reproductive function (as example, see Refs. 40 and 48), it remains possible that some regulatory mechanisms may not be totally similar between N6 cells and hypothalamic neurons. Nonetheless, the fact that leptin, insulin, and NPY appeared to evoke similar responses in terms of KiSS-1 gene expression in murine N6 cells and different *in vivo* models (22, 23) (present results) further support the validity of this clonal cell line as a complementary approach in studies on the regulation of the hypothalamic KiSS-1/GPR54 system.

Admittedly, our procedure of exploring changes in KiSS-1 and GPR54 gene expression in whole hypothalamic preparations by means of qrtRT-PCR does not allow the dissection of nucleus-specific fluctuations in the levels of those mRNAs. Nonetheless, two major reasons prompted us to use this approach. First, qrtRT-PCR enabled us to screen a large number of samples in a wide array of animal and cellular models. Second, as stated above, it is well established that in the male mouse, most of KiSS-1 gene expression at the hypothalamus is restricted to specific neuronal populations of arcuate nucleus (for a review, see Refs. 14, 16, and 19). Thus, we are confident that the observed responses in terms of KiSS-1 mRNA levels are representative of genuine changes in its expression at this specific hypothalamic site. In good agreement, studies using GNX *ob/ob* mice demonstrated that leptin-induced responses in hypothalamic KiSS-1 gene expression are detected at the arcuate nucleus. Notably, although the distribution of KiSS-1 mRNA within male mouse hypothalamus is now defined (14, 16), the pattern of expression of GPR54 remains less well characterized. Thus, although it has been described that a substantial proportion of GnRH neurons express GPR54 (46), it is possible that part of the changes in its mRNA levels reported herein might stem from other hypothalamic sites, an option that warrants further investigation.

In sum, we report herein a series of analyses on the regulation of the elements of the hypothalamic KiSS-1/GPR54 system by energy status and relevant metabolic cues in the mouse. By a combination of studies using *in vivo* models and the hypothalamic N6 cell line, our results further characterize the impact of changes in body energy stores on the hypothalamic expression of the KiSS-1 system and define the roles of key metabolic regulators, such as leptin and NPY, on the control of KiSS-1 and GPR54 expression in the mouse. Overall, our present findings reinforce the contention that the hypothalamic KiSS-1 system operates as a novel molecular conduit for the integration of energy balance, metabolism, and reproductive function.

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