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Developmental and Hormonally Regulated Messenger Ribonucleic Acid Expression of KiSS-1 and Its Putative Receptor, GPR54, in Rat Hypothalamus and Potent Luteinizing Hormone-Releasing Activity of KiSS-1 Peptide

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The gonadotropic axis is centrally controlled by a complex regulatory network of excitatory and inhibitory signals that is activated at puberty. Recently, loss of function mutations of the gene encoding G protein-coupled receptor 54 (GPR54), the putative receptor for the KiSS-1-derived peptide metastin, have been associated with lack of puberty onset and hypogonadotropic hypogonadism. Yet the pattern of expression and functional role of the KiSS-1/GPR54 system in the rat hypothalamus remain unexplored to date. In the present work, expression analyses of KiSS-1 and GPR54 genes were conducted in different physiological and experimental settings, and the effects of central administration of KiSS-1 peptide on LH release were assessed *in vivo*. Persistent expression of KiSS-1 and GPR54 mRNAs was detected in rat hypothalamus throughout postnatal development, with maximum expression levels at puberty in both male and female rats. Hypothalamic expression of KiSS-1 and GPR54 genes changed throughout the estrous cycle and was significantly increased after gonadectomy, a rise that was prevented by sex steroid replacement both in males and females. Moreover, hypothalamic expression of the KiSS-1 gene was sensitive to neonatal imprinting by estrogen. From a functional standpoint, intracerebroventricular administration of KiSS-1 peptide induced a dramatic increase in serum LH levels in prepubertal male and female rats as well as in adult animals. In conclusion, we provide novel evidence of the developmental and hormonally regulated expression of KiSS-1 and GPR54 mRNAs in rat hypothalamus and the ability of KiSS-1 peptide to potently stimulate LH secretion *in vivo*. Our current data support the contention that the hypothalamic KiSS-1/GPR54 system is a pivotal factor in central regulation of the gonadotropic axis at puberty and in adulthood. (*Endocrinology* 145: 4565–4574, 2004)

THE PITUITARY GONADOTROPINS, LH and FSH, are glycoprotein hormones primarily involved in the control of key gonadal functions such as ovulation, spermatogenesis, and sex hormone production (1). In mammals, LH and FSH secretion is controlled by a complex neuroendocrine network integrating central and peripheral signals. The pivotal hierarchical factor in the central control of gonadotropin secretion is the hypothalamic decapeptide LH-releasing hormone (LHRH), also termed GnRH, which dictates the pulsatile release of LH and FSH from pituitary gonadotropes (2, 3). In turn, episodic release of LHRH is exquisitely governed by the interplay of a plethora of excitatory and inhibitory signals at the hypothalamus (2, 3). In addition, gonadal hormones (sex steroids and peptides) and other peripheral signals participate in fine regulation of the gonadotropic axis, acting at hypothalamic and/or pituitary levels (2–4). Attainment of reproductive capacity at adulthood is the end point of a cascade of sex developmental events that leads to puberty. It is assumed that awakening of the neuroendocrine system controlling gonadotropin secretion at puberty is the result of the concerted enhancement of excitatory signals and the lowering of inhibitory inputs on LHRH neurons. However, despite recent developments in the field (for review, see Refs. 5–7), the primary mechanisms for such a maturational switch remain largely unknown.

Recently, loss of function mutations of the gene encoding G protein-coupled receptor 54 (GPR54) were shown to be unexpectedly associated with lack of puberty onset and hypogonadotropic hypogonadism in both humans and rodents (8, 9). GPR54 was initially cloned in the rat as an orphan receptor with 45% sequence similarity to galanin receptors (10). Thereafter, the human ortholog of GPR54, termed AXOR12 or hOT7T175, was identified (11–13). A search for the natural ligand(s) of this receptor identified a 54-amino acid secreted peptide, derived from the proteolytic processing of the product of the metastasis suppressor gene KiSS-1, with high affinity binding for GPR54 (11–13). This novel

Abbreviations: AUC, Area under the curve; C_T, cycle threshold; DPN, diarylpropionitrile; EB, estradiol benzoate; ER, estrogen receptor; GPCR, G protein-coupled receptor; icv, intracerebroventricular; LHRH, LH-releasing hormone; ORX, orchidectomized, orchidectomy; OVX, ovariectomized, ovariectomy; PPT, propylpyrazoletriol; PRL, prolactin; T, testosterone.

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protein, named metastin, contains a C-terminal Arg-Phe-NH_2 sequence distinctive for the RFamide peptide family. To date, a number of KiSS-1-derived peptides have been identified (13). These have been globally termed kisspeptins, and include metastin (kisspeptin-54), kisspeptin-14, and kisspeptin-13. They all share a common C-terminal RFamide motif and exhibit equal biopotency at rat and human GPR54 (13). In addition, other C-terminal fragments of KiSS-1, such as kisspeptin-10 or KiSS-$1_{112–121}$, KiSS-$1_{114–121}$, and KiSS-$1_{94–121}$, display high affinity binding for GPR54 and are provided with biological activities in different cell lines and/or *in vivo* systems (12, 13).

Metastin was initially purified from human placenta (11). Thereafter, significant expression of the KiSS-1 gene was also demonstrated in the brain, especially at the hypothalamus and basal ganglia (12, 14). Similarly, GPR54 mRNA has been found in placenta, several areas of the central nervous system, pituitary, spinal cord, and pancreas (12, 14). Low circulating levels of metastin have been detected in human plasma, which increase dramatically during pregnancy (15). From a functional standpoint, metastin is provided with potent antimetastasis activity on some tumors, such as melanoma and papillary thyroid carcinoma (11, 16). In addition, it was recently proposed that KiSS-1 peptides might play a role in the regulation of trophoblast invasion (15) and the control of some endocrine systems (13). However, the actual physiological functions of KiSS-1-derived peptides remain largely unexplored. In this context, the demonstration that inactivating mutations of their putative receptor led to reproductive failure due to hypogonadotropic hypogonadism highlighted a previously unsuspected role for the KiSS-1/GPR54 system in the control of the gonadotropic axis (8, 9). Yet the biological effects, regulatory mechanisms, site(s) of action, and developmental pattern of expression of this system within the reproductive axis have not been explored to date. To initiate such an analysis, we report herein the expression profile of KiSS-1 and GPR54 genes in the rat hypothalamus at different developmental stages and experimental settings. In addition, the effects of central administration of a biologically active KiSS-1 peptide on LH release are studied *in vivo*.

Materials and Methods

Animals and drugs

Wistar rats bred in the vivarium of University of Cordoba were used. The day the litters were born was considered 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 d 21 of age in groups of five rats per cage with free access to pelleted food and tap water. Experimental procedures were approved by the Cordoba University ethical committee for animal experimentation and were conducted in accordance with the European Union normative for care and use of experimental animals. In all experiments the animals were killed by decapitation, and for experiments involving mRNA analysis (experiments 1–5), the hypothalamus was dissected out, as described in detail previously (17), by a horizontal cut about 2 mm in depth with the following limits: 1 mm anteriorly from the optic chiasm, the posterior border of mamillary bodies, and the hypothalamic fissures. Hypothalamic samples were immediately removed upon decapitation, frozen in liquid nitrogen, and stored at −80 C until processing for RNA analysis. 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). Estradiol benzoate (EB) and testosterone (T) were purchased from Sigma-Aldrich Corp. (St. Louis, MO). The selective agonist of estrogen receptor α (ERα), propylpyrazoletriol (PPT), and the potency-selective ERβ ligand, diarylpropionitrile (DPN) were obtained from Tocris Cookson Ltd. (Avonmouth, UK).

Experimental designs

In the first series of experiments, analysis of hypothalamic expression of KiSS-1 and GPR54 mRNAs was conducted at different stages of postnatal development. Thus, in experiment 1, hypothalamic samples were obtained from male rats at 1 d (n = 10), 5 d (n = 10), 10 d (n = 10), 15 d (n = 5), 20 d (n = 5), 30 d (n = 5), 45 d (n = 5), 75 d (n = 5), and 18 months (540 d; n = 5/group) postpartum, corresponding to the neonatal (1 and 5 d), infantile (10 and 15 d), prepubertal (20 and 30 d), pubertal (45 d), adult (75 d), and aged stages of postnatal maturation (5). Similarly, in experiment 2, hypothalamic tissue samples were obtained from female rats at 1 d (n = 10), 5 d (n = 10), 10 d (n = 10), 15 d (n = 5), 20 d (n = 5), 30 d (n = 5), and 60 d (n = 5/group) postpartum, corresponding to the neonatal (1 and 5 d), infantile (10 and 15 d), prepubertal (20 d), pubertal (30 d), and adult (60 d) stages of postnatal development (5). On the latter, estrous cyclicity was monitored by daily vaginal cytology in adult females, and only rats with at least two consecutive 4-d estrous cycles were used. Representative hypothalamic samples were obtained from adult cyclic females at proestrous (at 1000 and 1800 h), estrous (at 1000 h), diestrous d 1 (at 1000 h), and diestrous d 2 (at 1000 h) phases of the ovarian cycle.

In the next set of experiments, regulation of hypothalamic expression of KiSS-1 and GPR54 genes by gonadal factors was monitored in male and female rats. Thus, in experiment 3, adult (75 d old) males were bilaterally orchidectomized (ORX) under light ether anesthesia, and hypothalamic samples (n = 5/group) were obtained 2 wk after surgery. An additional group of ORX males (n = 5) was implanted with SILASTIC brand silicon tubing (Dow Corning, Midland, MI) elastomers (40 mm in length; inner diameter, 0.062 cm; exterior diameter, 0.125 cm) containing T, and hypothalami were sampled 2 wk after ORX. Similarly, in experiment 4, bilateral ovariectomy (OVX) was performed under ether anesthesia in adult (60 d old) cycling females at random stages of the estrous cycle. Two weeks after OVX, rats (n = 5/group) were injected sc daily for 3 d with 0.2 ml olive oil (used as vehicle), 25 μg synthetic EB, 1.5 mg of the selective ERα agonist PPT, 1.5 mg of the potency-selective ERβ ligand, DPN, or PPT plus DPN. The experimental protocol and doses for the different ER ligands were selected based on previous references, including data from our group (18, 19). In addition, in experiment 5, the effects of neonatal exposure to estrogen on the hypothalamic expression levels of KiSS-1 and GPR54 mRNAs were evaluated. In the rat, estrogenization during critical periods of sexual differentiation of the hypothalamus (*i.e.* the perinatal period) has been reported to permanently impair functioning of the reproductive axis and puberty onset (20). In this setting, 1-d-old male rats were injected sc with a single dose of EB (500 μg/rat; dissolved in 100 μl olive oil), a regimen that has been reported to induce complete estrogenization in the male rat without major systemic toxicity (20). Vehicle (oil)-injected animals served as controls. The animals (n = 6/group) were killed on d 60 postpartum.

Finally, in experiment 6, the ability of KiSS-1 peptide to centrally modulate LH secretion was assessed *in vivo*. To this end, mouse KiSS-$1_{110–119}$-NH_2 peptide was used. This peptide is the rodent homologue of human KiSS-$1_{112–121}$-NH_2 or kisspeptin 10, which was previously shown to maximally bind and activate GPR54 in transfected Chinese hamster ovary cells (12, 13). Central intracerebroventricular (icv) administration of KiSS-1 peptide was conducted in prepubertal male (30 d old) and female (25 d old) rats as well as in adult (75 d old) males (n = 10–12 rats/group) as previously described (21, 22). A dose of 1 nmol KiSS-1 in 10 μl was injected per rat on the basis of previous references testing the neuroendocrine actions of different centrally administered peptides (22, 23). Groups of animals (n = 10–12) were sequentially killed 15 and 60 min after icv injection. Animals injected with vehicle (physiological saline, 0.9% NaCl) served as controls. Upon decapitation, trunk blood was collected, and serum samples were separated by centrifugation at 1600 × *g* for 20 min and stored at −20 C until use for hormone determinations (see below). As our initial data evidenced a potent LH-releasing effect of centrally administered KiSS-1 peptide, a detailed time-course analysis of such a response was conducted in experiment 7.

Thus, 1 nmol KiSS-1 peptide was icv injected into adult male rats (n = 10–12 rats/group), and systemic blood samples (300 μl) were obtained by jugular venipuncture before (0 min) and 15, 30, 45, 60, 90, 120, and 180 min after central administration of KiSS-1.

RNA analysis by semiquantitative RT-PCR

Total RNA was isolated from hypothalamic samples using the single-step, acid guanidinium thiocyanate-phenol-chloroform extraction method (24). Hypothalamic expression of KiSS-1 and GPR54 mRNAs was assessed by RT-PCR, optimized for semiquantitative detection, using the primer pairs and conditions indicated in Table 1. For each target, RT-PCR amplification was routinely conducted using two different sets of primers, which were generated on the basis of the published sequences of rat KiSS-1 and GPR54 genes (GenBank accession nos. AY196983.1 and NM023992.1, respectively) and designed to span the intron sequences. In addition, hypothalamic expression of LHRH mRNA was assessed in selected experimental groups using the primer pair and conditions described in Table 1. As an 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, as indicated in Table 1.

For amplification of the targets, 2 μg total RNA were used for RT-PCR in two consecutive separate steps. In addition, to enable appropriate amplification in the exponential phase for each target, PCR amplifications of specific signals and RP-S11 transcript were carried out in separate reactions with different number of cycles, but using similar amounts of the corresponding cDNA templates generated in single RT reactions, as previously described (25, 26). PCRs 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 products, 63.5 C for GPR54 products, and 58 C for LHRH and RP-S11 transcripts. Different numbers of cycles were tested to optimize amplification in the exponential phase of PCR. In detail, analysis of intensity of PCR signals as a function of the number of amplification cycles revealed a strong linear relationship among cycles 28–38 in the case of KiSS-1 and GPR54 transcripts (with correlation coefficients $r^2 \geq 0.97$) and among cycles 20–28 in the case of RP-S11 ($r^2 = 0.982$). On this basis, the numbers of PCR cycles indicated in Table 1 were chosen for additional semiquantitative analysis of specific targets and RP-S11 internal control.

PCR-generated DNA fragments were resolved in Tris-borate-buffered 1.5% agarose gels and visualized by ethidium bromide staining. The specificity of PCR products was confirmed by direct sequencing using a fluorescent dye termination reaction and an automated sequencer (Central Sequencing Service, University of Cordoba, Cordoba, Spain). Quantification of the 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 observed by final-time RT-PCR, real-time RT-PCR was performed in selected 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 representative hypothalamic samples from different stages of postnatal development in males (5-, 15-, 30-, 45-, and 75-d-old rats) and females (5-, 15-, 30-, and cyclic 60-d-old rats on diestrous d 1) and in neonatally estrogenized adult male rats. The synthesized cDNAs were further amplified (1/10th) 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 deoxy-NTPs, 3 mM $MgCl_2$, and 2.5 U iTaq DNA polymerase (Bio-Rad Laboratories) 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. Product purity was confirmed by dissociation curves and random agarose gel electrophoresis. No-template controls were included in all assays, yielding no consistent amplification. Calculation of the 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 iCycler iQ real-rime 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). For each experimental group assayed, reference samples were arbitrarily taken from 5-d-old rat hypothalamus (postnatal development) and control adult hypothalamic samples (estrogenization model). No significant differences in C_T values were observed for RP-S11 between the treatment groups.

Hormone measurement by specific RIA

Serum LH, prolactin (PRL), and GH levels were measured in a volume of 10–25 μl using a double-antibody method and RIA kits supplied by the NIH (Dr. A. F. Parlow, NIDDK National Hormone and Peptide

TABLE 1. Oligonucleotide primer pairs used for RT-PCR amplification of KiSS-1, GPR54 and RP-S11 transcripts in hypothalamic samples

Target	Oligo-primers	Expected size (bp)	PCR cycles
KiSS-1 (A)	KiSS-1 sense (5′-TGG CAC CTG TGG TGA ACC CTG AAC-3′) KiSS-1 antisense 1 (5′-ATC AGG CGA CTG CGG GTG GCA CAC-3′)	202	32
KiSS-1 (B)	KiSS-1 sense (5′-TGG CAC CTG TGG TGA ACC CTG AAC-3′) KiSS-1 antisense 2 (5′-GCC ACC TGC CTC CTG CCG TAG CGC-3′)	301	32
GPR54 (A)	GPR54 sense (5′-TGT GCA AAT TCG TCA ACT ACA TCC-3′) GPR54 antisense 1 (5′-AGC ACC GGG GCG GAA ACA GCT GC-3′)	194	32
GPR54 (B)	GPR54 sense (5′-TGT GCA AAT TCG TCA ACT ACA TCC-3′) GPR54 antisense 2 (5′-AGC AGG TAT AGG GCC AGC AGG TTG-3′)	303	32
LHRH	LHRH sense (5′-GCA CTA TGG TCA CCA GCG GG-3′) LHRH antisense (5′-CAT GGA TCT CAG CGT CAA TG-3′)	477	32
RP-S11	S11 sense (5′-CAT TCA GAC GGA GCG TGC TTA C-3′) S11 antisense (5′-TGC ATC TTC ATC TTC GTC AC-3′)	240	24

For KiSS-1 and GPR54 targets, two different primer combinations (A and B), whose sequences are included, were routinely used for analysis. The expected size of the generated cDNA products and the number of cycles selected for RT-PCR analysis are indicated for each signal.

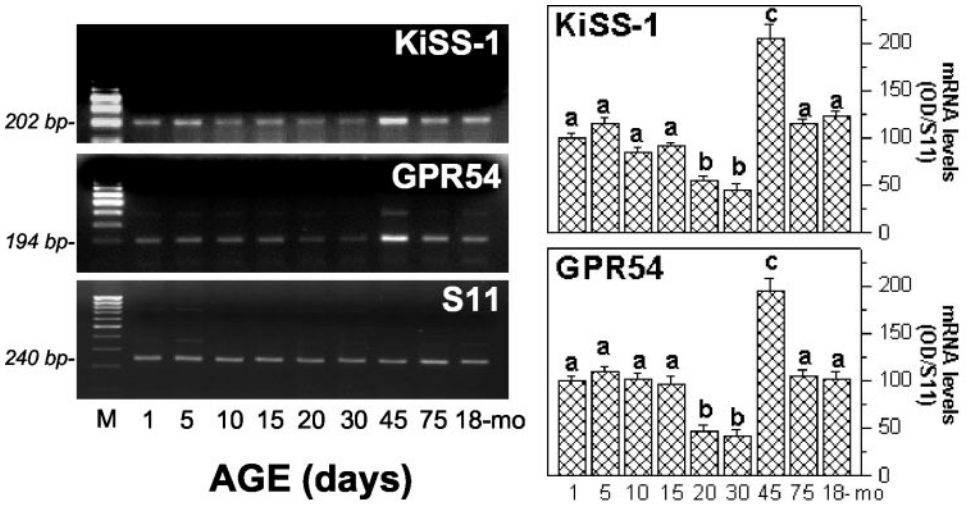

FIG. 1. Developmental profile of expression of KiSS-1 and GPR54 genes in rat hypothalamus throughout postnatal maturation in the male. In the *left panels*, representative RT-PCR assays are presented of expression levels of KiSS-1 and GPR54 mRNAs in hypothalamic samples from 1-, 5-, 10-, 15-, 20-, 30-, 45-, and 75-d-old as well as 18-month-old male rats. Parallel amplification of S-11 ribosomal protein mRNA served as internal control. In the *right panels*, semiquantitative values are the mean ± SEM of at least three independent determinations. Groups with *different superscript letters* are statistically different ($P < 0.05$, by ANOVA followed by Student-Newman-Keuls multiple range test).

Program, Bethesda, MD). Rat LH-I-9, PRL-I-6, and GH-I-7 were labeled with ^{125}I by the chloramine-T method, and the hormone concentrations were expressed using reference preparations LH-RP-3, PRL-RP-3, and GH-RP-2 as standards. Intra- and interassay coefficients of variation were less than 8% and 10%, respectively. The sensitivities of the assays were 20, 10, and 5 pg/tube for LH, PRL, and GH, respectively. The accuracy of hormone determination was confirmed by assessment of rat serum samples of known hormone concentrations used as external controls.

Presentation of data and statistics

Semiquantitative RT-PCR analyses were carried out in duplicate from at least three independent RNA samples of each experimental group. For generation of RNA samples, two or three hypothalamic fragments were pooled before isolation, and the generated samples were processed independently. Real-time RT-PCR analyses were conducted in triplicate. Semiquantitative RNA data are presented as the mean ± SEM. Serum LH determinations were conducted in duplicate, with a total number of 10–12 samples/determinations per group. Hormonal data are presented as the mean ± SEM. In addition, integrated LH secretory responses were expressed, when appropriate, as the area under the curve (AUC), calculated following the trapezoidal rule, over a 180-min period. Results were analyzed for statistically significant differences using ANOVA, followed by the Student-Newman-Keuls multiple range test (SigmaStat 2.0; Jandel Corp., San Rafael, CA). $P \leq 0.05$ was considered significant.

Results

KiSS-1 and GPR54 mRNA levels in rat hypothalamus during development and the estrous cycle

The profiles of hypothalamic expression of KiSS-1 and GPR54 mRNAs were first evaluated in samples from male rats at different stages of postnatal development, from the neonatal period to adulthood and aging. Stages of postnatal maturation were defined on the basis of previous references (for a review, see Ref. 5), and timing of puberty onset in male (45 d old) and female (30–35 d old) rats was defined on the basis of previously accepted criteria (5) and recording of external signs of reproductive maturation, such as vaginal opening and balanopreputial separation. Persistent expression of KiSS-1 and GPR54 mRNAs was detected in male rat hypothalamus by means of semiquantitative RT-PCR at all age points studied (1-, 5-, 10-, 15-, 20-, 30-, 45-, and 75-d-old rats as well as aged 18-month-old animals). However, hypothalamic levels of KiSS-1 and GPR54 mRNAs significantly changed, in a coordinated manner, during the study period; they were moderate at birth and up to d 15 postpartum, declined at the prepubertal stage (d 20 and 30 postpartum), and sharply increased thereafter, with maximum expression levels at puberty (45-d-old males; Fig. 1). Such an expression profile was confirmed by real-time RT-PCR analysis of representative hypothalamic samples that showed maximum expression levels in 45-d-old male rats (Table 2).

TABLE 2. Real-time RT-PCR data from triplicate analyses of hypothalamic KiSS-1 and GPR54 mRNA levels in representative experimental groups

Groups	mRNA levels (% over reference)	
	KiSS-1	GPR54
Male (5 d)	102.0 ± 6.0	105.0 ± 7.0
Male (15 d)	110.0 ± 7.0	112.0 ± 6.0
Male (30 d)	55.0 ± 4.0[a]	47.5 ± 5.0[a]
Male (45 d)	292 ± 17.5[a]	214.0 ± 12.5[a]
Male (75 d)	112.5 ± 9.0	115.0 ± 7.0
Female (5 d)	105.0 ± 8.0	100.0 ± 6.0
Female (15 d)	60.0 ± 6.0[a]	61.0 ± 5.0[a]
Female (30 d)	186.0 ± 10.0[a]	172.0 ± 9.0[a]
Female (60 d, diestrous d)	115.0 ± 7.0	105.0 ± 7.0
Control (60 d)	112.0 ± 8.0	102.0 ± 5.0
Neoestrogenized	30.9 ± 4.0[a]	115.0 ± 4.0

Representative samples from three settings were assayed: 1) different stages of postnatal development in males, 2) different stages of postnatal development in females, and 3) neonatally estrogenized male rats. Relative mRNA levels in the experimental groups are expressed as percentage over reference values, which were arbitrarily set at male 5-d (1), female 5-d (2), and control (3); *underlined*. Reference values were assigned a level of 100%; the other values were normalized accordingly. The percent expression over reference values was calculated for each group by the equation $2^{-\Delta\Delta CT}$.

[a] $P \leq 0.05$ *vs.* corresponding reference values (by ANOVA, followed by Student-Newman-Keuls multiple range test).

Similarly, hypothalamic expression of KiSS-1 and GPR54 mRNA was persistently detected in female rats throughout postnatal development, with moderate levels during the neonatal period, which decreased significantly in the prepubertal stage and maximally increased at puberty (30-d-old females; Fig. 2). Again, this expression profile was confirmed by real-time RT-PCR analysis of representative hypothalamic samples that showed maximum expression levels in

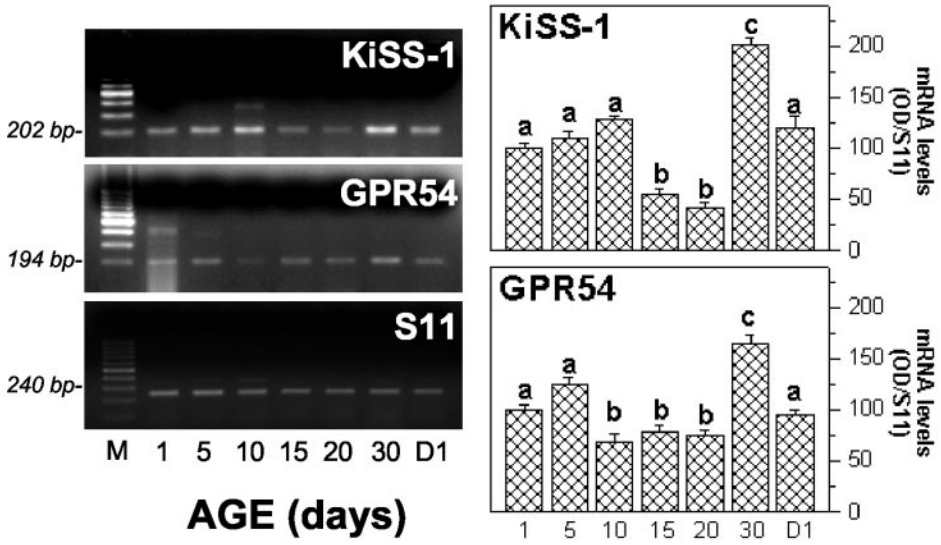

FIG. 2. Developmental profile of expression of KiSS-1 and GPR54 genes in rat hypothalamus throughout postnatal maturation in the female. In the *left panels*, representative RT-PCR assays are presented of expression levels of KiSS-1 and GPR54 mRNAs in hypothalamic samples from 1-, 5-, 10-, 15-, 20-, 30-, and 60-d-old female rats. In the latter, hypothalamic samples in the diestrous d 1 (D1) phase of the ovarian cycle were used. Parallel amplification of S-11 ribosomal protein mRNA served as an internal control. In the *right panels*, semiquantitative values are the mean ± SEM of at least three independent determinations. Groups with *different superscript letters* are statistically different ($P < 0.05$, by ANOVA followed by Student-Newman-Keuls multiple range test).

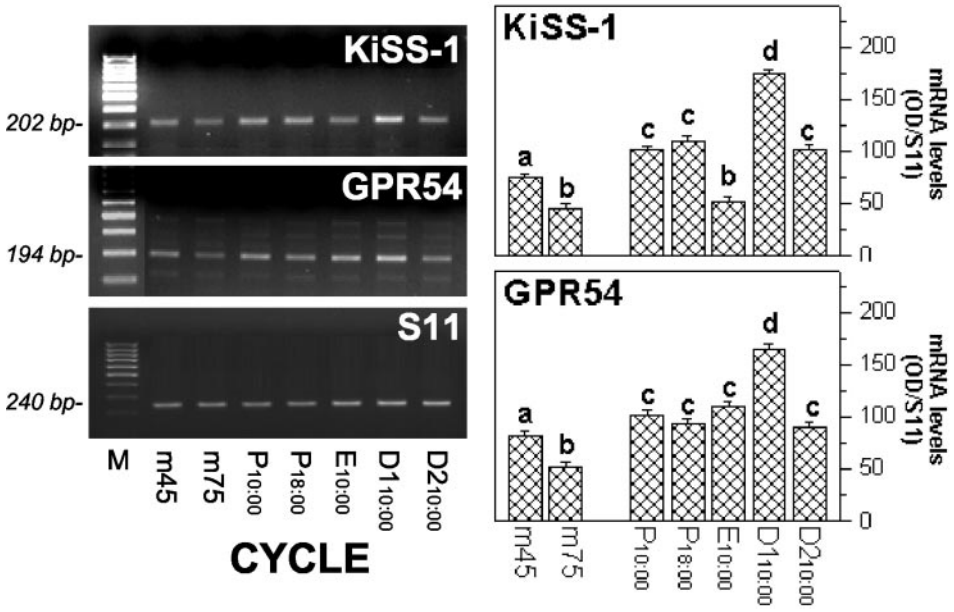

FIG. 3. Profile of expression of KiSS-1 and GPR54 genes in rat hypothalamus at different stages of the estrous cycle in the rat. In the *left panels*, a representative RT-PCR assay is presented of expression levels of KiSS-1 and GPR54 mRNAs in hypothalamic samples obtained from adult (60 d old) cyclic females at proestrous (P; at 1000 and 1800 h), estrous (E; at 1000 h), diestrous d 1 (D1; at 1000 h), and diestrous d 2 (D2; at 1000 h) phases of the ovarian cycle. For comparative purposes, expression analyses in 45- and 75-d-old male rats (m45 and m75) are also shown. Parallel amplification of S-11 ribosomal protein mRNA served as an internal control. In the *right panels*, semiquantitative values are the mean ± SEM of at least three independent determinations. Groups with *different superscript letters* are statistically different ($P < 0.05$, by ANOVA followed by Student-Newman-Keuls multiple range test).

30-d-old female rats (Table 2). In adult cyclic females, relative levels of KiSS-1 and GPR54 mRNAs throughout the estrous cycle were persistently higher than those in age-matched males. The expression levels of these transcripts significantly varied along the cycle, with peak levels on diestrous d 1 for KiSS-1 and GPR54 and low levels at estrus for KiSS-1 (Fig. 3).

Hypothalamic KiSS-1 and GPR54 mRNA levels after gonadectomy and estrogenization

Regulation of hypothalamic expression of KiSS-1 and GPR54 mRNAs by gonadal factors was evaluated using models of male and female gonadectomy. Because these experimental manipulations are known to induce dramatic changes in the functioning of the gonadotropic axis, serum LH levels and hypothalamic LHRH mRNA expression were also monitored. Orchidectomy (ORX) of adult animals resulted in a significant, clear-cut increase in hypothalamic KiSS-1 mRNA levels 2 wk after surgery, which was associated with a moderate rise in GPR54 mRNA expression (Fig. 4). In this setting, serum LH levels significantly increased 2 wk after ORX, whereas hypothalamic steady state levels of LHRH mRNA remained unaffected (Fig. 4). T replacement (40-mm SILASTIC brand elastomers containing T) blunted the rise in circulating LH levels and totally prevented the increase in hypothalamic KiSS-1 mRNA levels 2 wk after ORX. Similarly, T supplementation blocked the moderate increase in GPR54 mRNA after ORX and partially suppressed hypothalamic LHRH mRNA levels (Fig. 4).

In the female rat, hypothalamic levels of KiSS-1 and, to a lesser extent, GPR54 mRNAs significantly increased 2 wk after OVX. Estradiol replacement to OVX rats totally prevented such a response; a phenomenon that was mimicked by the administration of the selective agonist of ERα, PPT,

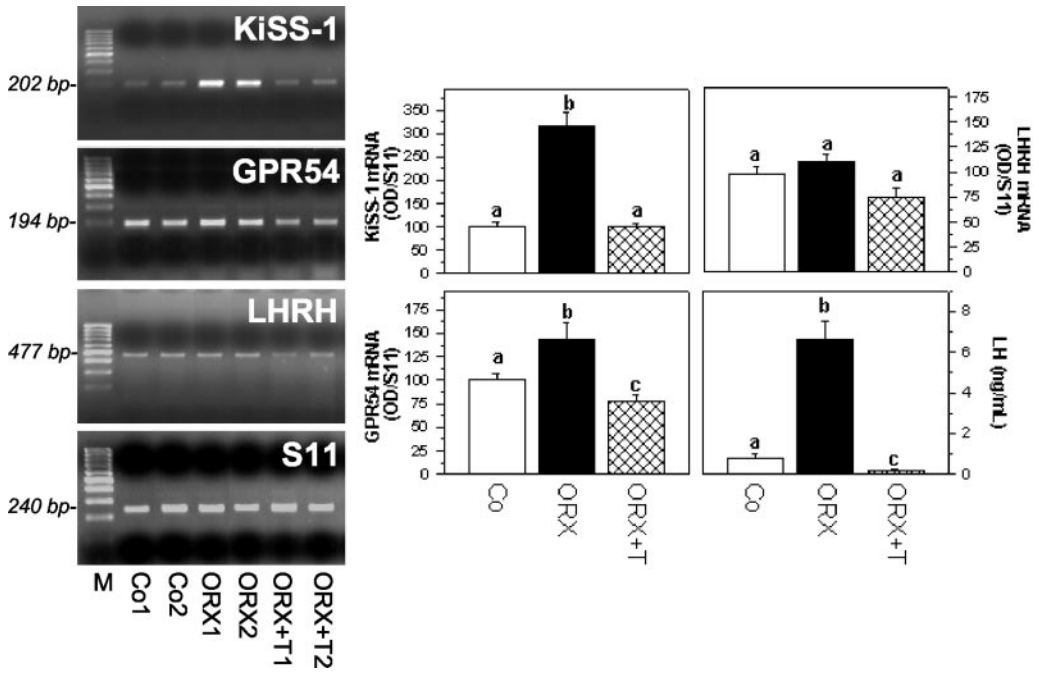

FIG. 4. Profile of expression of KiSS-1, GPR54, and LHRH genes in rat hypothalamus in adult (75 d old) rats, 2 wk after ORX with or without T replacement. In the *left panels*, a representative RT-PCR assay is presented of expression levels of the targets in hypothalamic samples from control males (Co), ORX rats, and ORX animals implanted with SILASTIC brand capsules containing T (ORX+T). Two independent samples per group are presented. Parallel amplification of S-11 ribosomal protein mRNA served as an internal control. In the *right panels*, semiquantitative values of gene expression levels are the mean ± SEM of at least three independent determinations. In addition, serum LH levels in the different experimental groups are presented. Groups with different superscript letters are statistically different ($P < 0.05$, by ANOVA followed by Student-Newman-Keuls multiple range test).

but not of the potency-selective ligand of ERβ, DPN. The responses to the combined administration of PPT and DPN were not significantly different from those of administration of PPT alone (Fig. 5). As was the case in males, serum LH levels in gonadectomized females changed in parallel to hypothalamic KiSS-1 transcript levels, with a significant increase in LH concentrations in OVX females that was prevented by administration of estradiol and PPT, but not DPN. In contrast, 2-wk OVX resulted in a significant decrease in hypothalamic LHRH mRNA levels that were stimulated by estradiol replacement as well as by PPT and DPN administration. For the latter, hypothalamic LHRH mRNA levels were maximally increased by administration of the potency-selective ERβ ligand DPN (Fig. 5).

In addition, the effects of neonatal estrogen exposure on the expression of KiSS-1, GPR54, and LHRH mRNAs in the hypothalamus and on serum LH levels were monitored in adult male rats. Inappropriate exposure to estrogenic compounds during critical periods of sex differentiation of the hypothalamus in the rat has been reported to induce a plethora of reproductive defects through mechanisms that remain partially unexplored (20). Neonatal administration of EB (500 μg/rat, on d 1 postpartum) induced a significant decrease in serum LH concentrations and hypothalamic KiSS-1 mRNA levels in 60-d-old animals. In contrast, hypothalamic expression of GPR54 and LHRH mRNAs remained unaffected in neonatally estrogenized adult males (Fig. 6). The profile of expression of KiSS-1 and GPR54 mRNAs after neonatal estrogenization was confirmed by real-time RT-PCR analysis of representative hypothalamic samples (Table 2).

Effects of icv administration of KiSS-1 peptide on LH secretion

To evaluate its role in the central control of the gonadotropic axis, the effects of icv administration of the active fragment of KiSS-1 peptide, mouse KiSS-$1_{110-119}$-NH_2, on LH secretion were tested in prepubertal and adult rats. In prepubertal animals, central injection of 1 nmol/rat KiSS-1 peptide elicited a clear-cut increase in serum LH levels in both males (30 d old) and females (25 d old), with maximal stimulation (~10- to 12-fold increase over controls) 15 min after injection. In contrast, serum PRL levels were moderately inhibited by central administration of KiSS-1 peptide to prepubertal animals. Likewise, in adult male rats, icv injection of 1 nmol KiSS-1 peptide induced a significant increase in serum LH levels 15 and 60 min after administration. In this setting, serum PRL levels remained unaffected after central administration of KiSS-1 peptide (Fig. 7). Serum GH levels were not modified by icv injection of KiSS-1 (1 nmol/rat) in either prepubertal males (1.1 ± 0.26 ng/ml at 15 min after KiSS-1 *vs.* 1.85 ± 0.6 ng/ml in controls) or prepubertal females (0.84 ± 0.14 ng/ml at 15 min after KiSS-1 *vs.* 0.81 ± 0.16 ng/ml in controls). Because our initial data evidenced a very potent LH releasing activity of KiSS-1, a detailed time-course analysis of the LH response to icv injection of 1 nmol/rat KiSS-1 was conducted in adult animals. Central injection of KiSS-1 resulted in a dramatic increase in serum LH levels over basal (0 min) values that peaked 15 min after peptide injection. Moreover, serum LH concentrations in KiSS-1-injected animals were higher than those in paired vehicle-

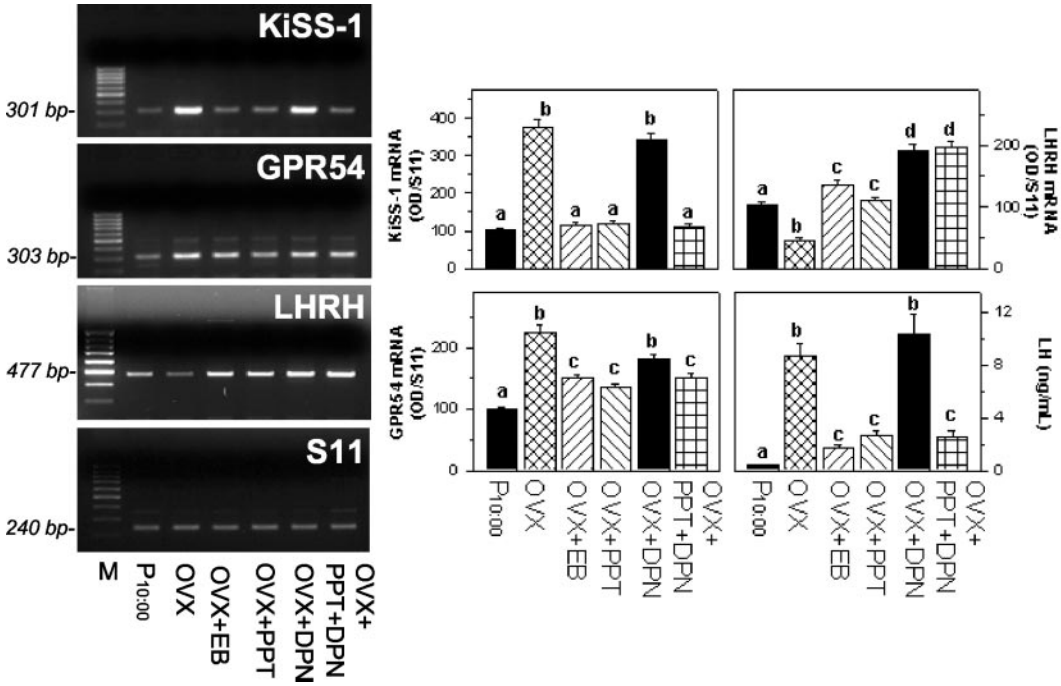

FIG. 5. Profile of expression of KiSS-1, GPR54, and LHRH genes in rat hypothalamus in adult (60 d old) rats, 2 wk after OVX with or without estrogen replacement, or administration of the selective ERα agonist PPT, the potency-selective ERβ ligand DPN, or PPT plus DPN to OVX rats. Parallel amplification of S-11 ribosomal protein mRNA served as an internal control. In the *right panels*, semiquantitative values of gene expression levels are the mean ± SEM of at least three independent determinations. In addition, serum LH levels in the different experimental groups are presented. Groups with *different superscript letters* are statistically different ($P < 0.05$, by ANOVA followed by Student-Newman-Keuls multiple range test).

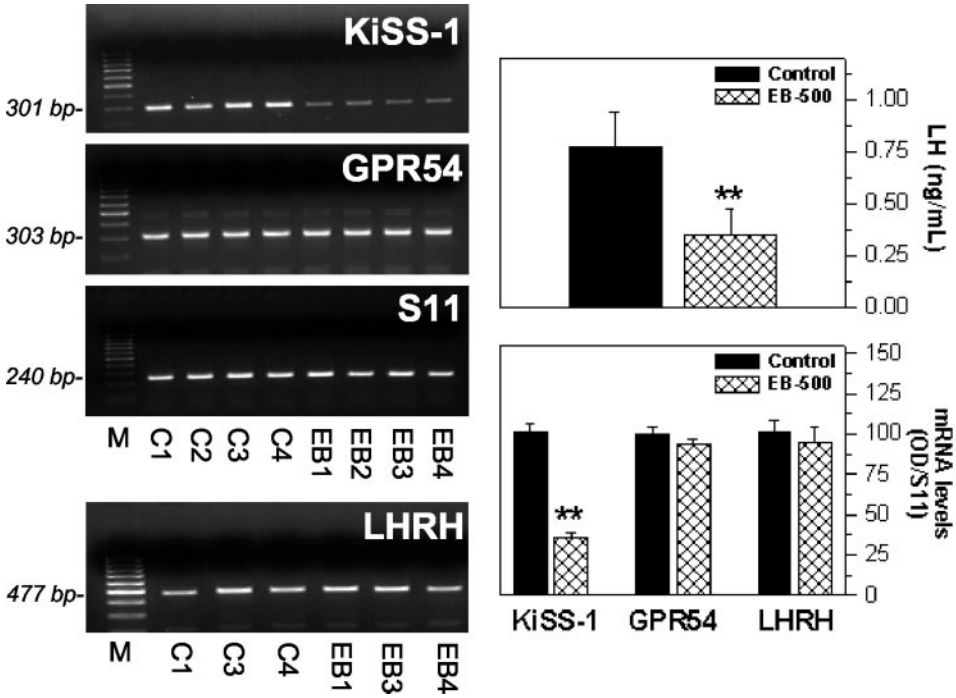

FIG. 6. Hypothalamic profile of expression of KiSS-1, GPR54, and LHRH genes in neonatally estrogenized adult (60 d old) male rats. In the *left panels*, representative RT-PCR assays are shown of expression levels of KiSS-1, GPR54, and LHRH mRNAs in hypothalamic samples obtained from control males (C1–C4) and neonatally estrogenized male rats (EB1–EB4). Four independent samples per group are presented. Parallel amplification of S-11 ribosomal protein mRNA served as an internal control. In the *right panels*, semiquantitative values of gene expression levels are the mean ± SEM of at least four independent determinations. In addition, serum LH levels in the different experimental groups are presented. **, $P < 0.05$ *vs.* corresponding controls (by ANOVA followed by Student-Newman-Keuls multiple range test).

injected animals during the 180-min study period following drug administration. Accordingly, integrated LH secretion during this time frame (180 min), as estimated by the AUC, was significantly increased by central icv injection of 1 nmol KiSS-1 (Fig. 8).

Discussion

Recently, a role for the KiSS-1/GPR54 system in control of the gonadotropic axis and puberty onset has emerged (8, 9). Yet the biological effects, regulatory mechanisms,

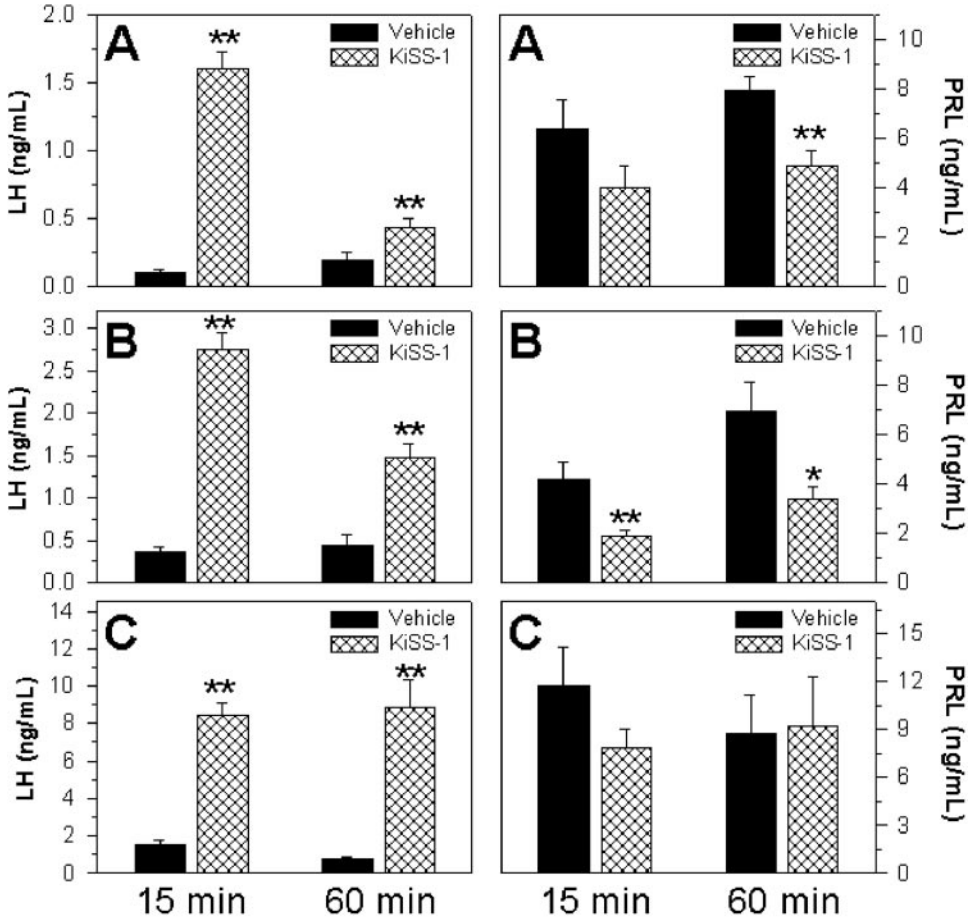

FIG. 7. Serum LH and PRL levels in prepubertal 30-d-old males (A), prepubertal 25-d-old females (B), and adult 75-d-old males (C) at 15 and 60 min after central icv administration of vehicle or 1 nmol/rat KiSS-1 in 10 μl. **, $P < 0.01$ *vs.* corresponding vehicle-injected controls (by ANOVA followed by Student-Newman-Keuls multiple range test).

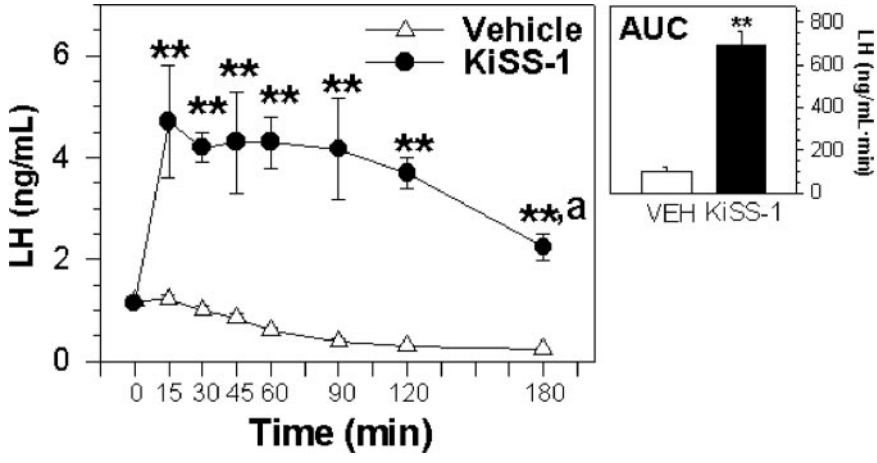

FIG. 8. Serum LH levels in adult (75 d old) male rats before (0 min) and 15, 30, 45, 60, 90, 120, and 180 min after central icv administration of vehicle or 1 nmol/rat KiSS-1 in 10 μl. In addition to the profiles of mean LH levels in the experimental groups, the integrated secretory responses, as the AUC over the study period (180 min), are shown. **, $P < 0.01$ *vs.* corresponding vehicle-injected controls; a, $P < 0.01$ *vs.* KiSS-1 injected group at 15 min (by ANOVA followed by Student-Newman-Keuls multiple range test).

site(s) of action, and developmental pattern of expression of this system within the reproductive axis have not been explored to date. To our knowledge, this is the first study to report the developmental and hormonally regulated pattern of expression of the genes encoding KiSS-1 and its putative receptor, GPR54, in rat hypothalamus. Our expression analyses during postnatal development showed that the lowest hypothalamic mRNA levels of KiSS-1 and GPR54 occur during the prepubertal stage, whereas maximum expression of these genes was observed at puberty in both male and female rats. Despite low circulating levels of pituitary gonadotropins before puberty, prepubertal LHRH neurons are intrinsically able to increase their secretory activity after certain experimental manipulations, such as electrical stimulation or administration of *N*-methyl-D-aspartate, an agonist of ionotropic excitatory amino acid receptors (5). Thus, it is evident that puberty onset is mainly due to activation of central excitatory inputs during the juvenile-pubertal transition period. Among others, neuronal systems using the excitatory

amino acids, noradrenaline and neuropeptide Y, have been involved in such phenomena (4, 5). Our current data suggest that, in addition, enhanced expression of both components of the KiSS-1/GPR54 system at the hypothalamus might contribute to activation of the gonadotropic axis at puberty. This contention is supported by our functional *in vivo* data, because central administration of KiSS-1 peptide to prepubertal male and female rats was able to significantly increase serum LH levels from low prepubertal values to levels similar to those in adult animals. Nevertheless, although the above data might indicate a role for the hypothalamic KiSS-1 system as a trigger of puberty onset, a permissive action of this novel factor in puberty development cannot be excluded on the basis of our current results. Experiments involving chronic central administration of KiSS-1 peptide to immature animals are presently in progress in our laboratory to address this issue.

Additional evidence for the involvement of the hypothalamic KiSS-1/GPR54 system in the regulation of the gonadotropic axis is indirectly provided by its modulation by gonadal factors. Expression levels of KiSS-1 and GPR54 mRNAs significantly varied during the estrous cycle, with peak levels on diestrous d 1 for KiSS-1 and GPR54 and low levels at estrus for KiSS-1. This observation strongly suggests that circulating gonadal hormones might participate in regulation of the hypothalamic expression of KiSS-1 and GPR54 genes. Accordingly, gonadectomy, in both males and females, resulted in a significant increase in KiSS-1 and, to a lesser extent GPR54, mRNA expression at the hypothalamus. These responses can be attributed to the removal of sex steroid inhibitory inputs, because they were prevented by replacement of ORX and OVX animals with T and EB, respectively. Moreover, activation of ERα by the selective ligand PPT, but not ERβ, by the potency-selective agonist DPN was able to mimic the effects of estrogen supplementation in OVX rats, thus suggesting the involvement of ERα pathways in this phenomenon. The above responses in terms of hypothalamic gene expression in the different experimental groups closely paralleled the changes in serum LH levels after gonadectomy and hormonal replacement. In contrast, transcriptional regulation of the LHRH gene at the hypothalamus did not clearly follow changes in circulating LH values. Taken together, our data strongly suggest that the hypothalamic KiSS-1/GPR54 system may play a role not only in activation of the gonadotropic axis at puberty, but also in its regulation in adulthood. Moreover, our present results suggest that the effects of KiSS-1 on hypothalamic LHRH, if any, are apparently not conducted at the transcriptional level. Alternatively, LHRH-independent actions of KiSS-1 in the control of LH secretion (*e.g.* directly at the pituitary level, acting as a hypophysiotropic neuropeptide) cannot be excluded. These phenomena are currently under investigation at our laboratory.

In addition to acute regulation by gonadal hormones, hypothalamic expression of KiSS-1 mRNA appears to also be sensitive to the organizing effects of neonatal estrogen. Acting at critical periods of sex development, estrogen has been involved in the functional organization of the hypothalamic-pituitary unit responsible for the control of gonadotropin secretion throughout the life span (20). In the male rat, locally produced estrogen after aromatization of testis-derived T promotes hypothalamic masculinization during a developmental frame that spans from embryonic d 17.5 to d 10 postpartum (20). Indeed, key events in hypothalamic function, such as the expression of ERα and ERβ genes (28), are imprinted by neonatal estrogen. Our current data point out that the neonatal endocrine (estrogen) milieu can also imprint the pattern of expression of the KiSS-1 gene in male rat hypothalamus. In fact, relative mRNA levels of KiSS-1 at the hypothalamus were persistently suppressed in adults by neonatal estrogenization. In contrast, hypothalamic expression of neither GPR54 nor LHRH genes was altered in adulthood by neonatal exposure to high doses of estrogen. Again, changes in LH levels closely paralleled those in hypothalamic KiSS-1 mRNA levels, because neonatal exposure to estrogen also induced a persistent decrease in serum LH concentrations in adult male rats. Although this association may not be causative, the contribution of persistently decreased KiSS-1 expression at the hypothalamus to the plethora of developmental and functional defects in the gonadotropic axis after neonatal exposure to supraphysiological doses of estrogen merits further investigation.

Central administration of KiSS-1 peptide elicited a very potent secretory response in terms of LH secretion in prepubertal and adult animals. In the latter, a strikingly long-lasting response to central icv administration of 1 nmol KiSS-1 was observed in time-course analysis over a 180-min period. Such an LH-releasing effect was not unspecific, because serum GH levels remained unaffected, and serum PRL levels were moderately inhibited by icv injection of KiSS-1. The fact that central injection of KiSS-1 peptide was able to potently stimulate LH secretion before puberty and in adult males also supports the contention that this system may play a relevant role in the regulation of the gonadotropic axis in adulthood. This phenomenon has been previously demonstrated for other hypothalamic systems, such as erbB-2/erbB-4, which has been involved not only in puberty onset, but also in regulation of the reproductive axis in the adult cyclic female rat (29). As indicated above, a moderate decrease in serum PRL levels was detected after icv injection of KiSS-1 to prepubertal rats. Although a rise in serum PRL levels is detected during puberty in the rat (5), high PRL levels are frequently associated with low gonadotropin secretion. Thus, it tempting to speculate that the combined stimulatory and inhibitory effects of hypothalamic KiSS-1 on LH and PRL secretion, respectively, might be relevant for maximal activation of the gonadotropic axis at puberty onset. The hypothalamic targets and molecular mechanisms by which central administration of KiSS-1 peptide modulates LH (and eventually PRL) secretion are presently under investigation. In this context, detailed characterization of the pattern of expression of both components (ligand and receptor) of the KiSS-1/GPR54 system within the hypothalamus will help to characterize the mode of action of KiSS-1 in neuroendocrine control of the reproductive axis.

In summary, our current data point out that the genes encoding KiSS-1 peptide and its putative receptor, GPR54, are expressed in the rat hypothalamus in a developmental and hormonally regulated manner (by gonadally derived factors), and that central administration of KiSS-1 peptide is

able to selectively and potently elicit LH secretion in prepubertal and adult animals. These data suggest that hypogonadotropic hypogonadism associated with null mutations of the GPR54 gene in both the mouse and human is at least partially due to the blockade of KiSS-1 actions at the hypothalamus. Nevertheless, the possibility that the abnormal gonadal phenotype in GPR54 knockout mice might partially derive from primary defects of KiSS-1 actions at other levels of the reproductive axis (*i.e.* pituitary and gonads) cannot be ruled out. Overall, our present results indicate that the KiSS-1/GPR54 system is a novel member of the complex regulatory network of excitatory signals involved in the central control of gonadotropin secretion and support the contention that this system is a pivotal factor in regulation of the gonadotropic axis at puberty and in adulthood.

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