0013-7227/05/$15.00/0
Printed in U.S.A.

Endocrinology 146(1):156–163
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doi: 10.1210/en.2004-0836

Characterization of the Potent Luteinizing Hormone-Releasing Activity of KiSS-1 Peptide, the Natural Ligand of GPR54

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Loss-of-function mutations of the gene encoding GPR54, the putative receptor for the KiSS-1-derived peptide metastin, have been recently associated with hypogonadotropic hypogonadism, in both rodents and humans. Yet the actual role of the KiSS-1/GPR54 system in the neuroendocrine control of gonadotropin secretion remains largely unexplored. To initiate such analysis, the effects of KiSS-1 peptide on LH secretion were monitored using *in vivo* and *in vitro* settings under different experimental conditions. Central intracerebroventricular administration of KiSS-1 peptide potently elicited LH secretion *in vivo* over a range of doses from 10 pmol to 1 nmol. The effect of centrally injected KiSS-1 appeared to be mediated via the hypothalamic LHRH. However, no effect of central administration of KiSS-1 was detected on relative LHRH mRNA levels. Likewise, systemic (ip and iv) injection of KiSS-1 markedly stimulated LH secretion. This effect was similar in terms of maximum response to that of central administration of KiSS-1 and might be partially attributed to its ability to stimulate LH secretion directly at the pituitary. Finally, the LH-releasing activity of KiSS-1 was persistently observed after blockade of endogenous excitatory amino acid and nitric oxide pathways, *i.e.* relevant neurotransmitters in the neuroendocrine control of LH secretion. In summary, our results provide solid evidence for a potent stimulatory effect of KiSS-1 on LH release, acting at central levels (likely the hypothalamus) and eventually at the pituitary, and further document a novel role of the KiSS-1/GPR54 system as a relevant downstream element in the neuroendocrine network governing LH secretion. (*Endocrinology* 146: 156–163, 2005)

THE G PROTEIN-coupled receptor GPR54 was originally identified in the rat as an orphan receptor with 45% sequence similarity to galanin receptors (1). Thereafter, the human ortholog of GPR54, termed AXOR12 or hOT7T175, was cloned (2–4). The natural ligand of this receptor, termed metastin, was found to derive from the proteolytic processing of the product of the metastasis suppressor gene KiSS-1 (2–4). Metastin is a 54-amino acid secreted peptide, which contains a C-terminal Arg-Phe-NH_2 sequence distinctive of the RFamide peptide family. To date, a number of additional KiSS-1-derived peptides, structurally related to metastin and globally named kisspeptins, have been identified (4). They all share a common C-terminal RFamide motif and exhibit equal biopotency at rat and human GPR54 (3, 4). In terms of biological functions, KiSS-1 is provided with potent antimetastasis activity in some tumors, such as papillary thyroid carcinoma, breast carcinoma, melanoma, and pancreatic cancer cells (2, 5–7), and loss of KiSS-1 gene expression has been linked to tumor progression and metastasis in esophageal squamous cell carcinoma and bladder cancer (8, 9). In addition, in keeping with its ability to inhibit migration of cancer cells, it was recently proposed that KiSS-1 peptides likely play a role in the physiological regulation of trophoblast invasion (10). Moreover, some preliminary evidence suggested that KiSS-1 might participate in the regulation of specific neuroendocrine systems (*e.g.* oxytocin release) (4). Indeed, expression of KiSS-1 and/or GPR54 has been demonstrated in a variety of normal tissues, including placenta, different brain areas (specially hypothalamus and basal ganglia), spinal cord, pituitary, pancreas, and human plasma (2, 3, 11, 12), which strongly suggests additional, as-yet-unknown physiological roles of this newly discovered system.

In this context, an unexpected reproductive facet of the KiSS-1/GPR54 system has recently emerged (11, 13, 14). Thus, two independent reports recently provided conclusive evidence demonstrating that a number of point mutations and deletions of the GPR54 gene are found in patients suffering familiar forms of idiopathic hypogonadotropic hypogonadism (13, 14). This clinical syndrome was reproduced in mouse models carrying null mutations of the GPR54 gene (11, 14). Accordingly, the KiSS-1/GPR54 system was proposed to play a previously unsuspected, relevant role in the regulation of the development and/or function of the hypothalamic-pituitary-gonadal axis. Yet the regulatory mech-

First Published Online September 16, 2004

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Abbreviations: AMPA, 2-Amino-3-hydroxy-5-methyl-4-isoxazol propionic acid; AUC, area under the curve; EAA, excitatory amino acid; icv, intracerebroventricular; KA, kainate; NAME, *N*-Nitro$_w$-arginine-methyl ester; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; NOS, NO synthase; POA, preoptic area; RT, reverse transcription.

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anisms, site(s) of action, and biological effects of this system within the reproductive axis remain so far mostly unexplored. In fact, despite their central role in the control of reproductive function (15), detailed characterization of the role of the KiSS-1/GPR54 system in the neuroendocrine network governing the secretion of pituitary gonadotropins is still pending. In this sense, our recent data evidenced that hypothalamic expression of KiSS-1 and GPR54 genes is maximum at puberty and under the regulation of sex steroids and preliminarily demonstrated a potent LH-releasing activity of intracerebroventricular administration of KiSS-1 peptide in the rat (16). Likewise, Gottsch *et al.* (17) very recently reported the LH releasing action of KiSS-1 peptide in the mouse. In good agreement, data from mouse models carrying null mutations of the GPR54 gene demonstrated defective LH content and preserved response to LHRH at the pituitary (14). In this context, the present experimental work was undertaken to delineate the site(s) and mode of action of KiSS-1 in the control of pituitary LH secretion. To this end, the LH-releasing activity of KiSS-1 peptide was monitored in different *in vivo* and *in vitro* settings under different experimental conditions.

Materials and Methods

Animals and drugs

Wistar rats bred in the vivarium of the University of Córdoba were used. The day the litters were born was considered d 1 of age. The animals were maintained under constant conditions of light (14 h of light, from 0700 h) and temperature (22 C) and were weaned at 21 d of age in groups of five rats per cage with free access to pelleted food and tap water. Experimental procedures were approved by the Córdoba University Ethical Committee for Animal Experimentation and were conducted in accordance with the European Union normative for care and use of experimental animals. 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). The decapeptide LHRH was purchased from Sigma Chemical Co. (St. Louis, MO), and the potent LHRH antagonist Org 30276 (Ac-D-pClPhe-D-pClPhe-D-Trp-Ser-Tyr-D-Arg-Leu-Arg-Pro-D-Ala-NH_2CH_3COOH) was generously supplied by Organon (Oss, The Netherlands). The antagonist of ionotropic *N*-methyl-D-aspartate (NMDA) receptors, MK-801, and the antagonist of kainate (KA) and 2-amino-3-hydroxy-5-methyl-4-isoxazol propionic acid (AMPA) receptors, NBQX, were purchased from Research Biochemicals International (Natick, MA). The inhibitor of nitric oxide (NO) synthases (NOSs), *N*-nitro$_w$-arginine-methyl ester (NAME) was obtained from Sigma.

Experimental designs

On the basis of our preliminary data showing a potent LH-releasing activity of KiSS-1 (16), in experiment 1 a detailed dose-response analysis of the effects of centrally administered KiSS-1 was conducted in pubertal (45 d old) male rats. This age point was selected, given the proposed key role of the KiSS-1/GPR54 system in the control of puberty onset (14). To this end, groups of males (n = 10) were implanted with intracerebroventricular (icv) cannulae, as described in detail elsewhere (18), and KiSS-1 was centrally injected over a range of doses from 1 nmol to 10 pmol in 10 μl. The highest dose was selected on the basis of our initial data on the ability of 1 nmol KiSS-1 to potently elicit LH secretion (16) and previous references testing the neuroendocrine actions of different centrally administered peptides (18, 19). Pair-aged males (n = 10) injected with vehicle (physiological saline; NaCl 0.9%) served as controls. Trunk blood samples were taken on decapitation of the animals at 15 and 60 min after KiSS-1 injection for hormone determination.

Because results from experiment 1 evidenced a potent stimulatory action of centrally injected KiSS-1 on LH secretion, the next experiments aimed at identifying the potential interplay between KiSS-1 and hypothalamic LHRH. Thus, in experiment 2, pubertal (45 d old) male rats (n = 10–12/group) were twice sc injected with a potent LHRH antagonist (5 mg/kg·24 h; two doses) to completely block endogenous LHRH actions. A detailed time-course analysis of serum gonadotropin levels after single administration of this dose of the antagonist has been previously reported, showing maximum suppression of circulating LH levels 24 h after injection of the analog (20). Vehicle-injected groups served as controls. Twenty-four hours after the last dose of the antagonist, the animals were icv injected with 1 nmol KiSS-1 or vehicle, and trunk blood samples were collected 15 min later. In addition, in experiment 3, the effects of combined administration of KiSS-1 and LHRH on LH secretion were evaluated. Pubertal (45 d old) male rats were simultaneously injected with LHRH (1 μg/rat, equivalent to 0.85 nmol; ip) and KiSS-1 (1 nmol/rat; icv). Vehicle-injected groups served as controls. Trunk blood samples were collected 15 min after administration of the peptides. Finally, the effects of centrally administered KiSS-1 peptide on LHRH gene expression were monitored in experiment 4. In detail, two experimental settings were used. Firstly, peripubertal (35 d old) male rats were chronically icv injected with KiSS-1 for 7 d at a dose of 1 nmol KiSS-1/12 h. On decapitation of the animals, hypothalamic samples were obtained 60 min after the last injection of KiSS-1. Second, early adult (60 d old) male rats were icv injected with a single dose of 1 nmol KiSS-1, and hypothalamic samples were collected 15, 60, and 180 min later. Hypothalamic tissue [including the preoptic area (POA)] was dissected out, as described in detail elsewhere (21), by a horizontal cut of about 2-mm depth with the following limits: 2 mm anteriorly from the optic chiasm, the posterior border of mamillary bodies, and the hypothalamic fissures. Hypothalamic samples were immediately removed on decapitation, frozen in liquid nitrogen, and stored at −80 C until processing for RNA analysis.

In the next series of experiments, the effects of systemic administration of KiSS-1 peptide on LH secretion were monitored. In experiment 5, KiSS-1 (7.5 nmol/rat; equivalent to 10 μg/animal) was administered ip to pubertal (45 d old) male rats, and trunk blood samples were obtained at 15 and 60 min. For comparative purposes, pair-aged males were icv injected with 1 nmol KiSS-1, as described in previous experiments. In addition, in experiment 6, the effect of iv injection of KiSS-1 on LH release was monitored in freely moving rats. To this end, groups of male rats (n = 6) were implanted with intracardiac cannulae, as described in detail elsewhere (22), and blood samples (250 μl) were taken every 15 min over a 240-min period. For proper handling, the animals were sampled four times before iv injection of KiSS-1 (7.5 nmol/rat) or vehicle. Finally, because data from the above experiments evidenced a clear stimulatory effect of systemic administration of KiSS-1, the ability of the peptide to modulate LH secretion directly at the pituitary level was addressed in experiment 7, using static incubations of pituitary tissue from pubertal (45 d old) male rats. Procedures for incubation of pituitary samples have been described in detail elsewhere (18, 22). Briefly, on decapitation of the animals, anterior pituitaries (n = 10–12 per group) were removed and placed in scintillation vials in a Dubnoff shaker at 37 C with constant shaking (60 cycles/min) under an atmosphere of 95% O_2-5% CO_2. After 1 h of preincubation, the media were replaced by either fresh medium alone (DMEM) or medium containing increasing doses of KiSS-1 (10^{-10}, 10^{-8}, 10^{-6} M). For comparative purposes, a group of pituitary samples was incubated with LHRH (10^{-8} M). Samples from the incubation media were collected at 60 and 180 min for hormone determinations.

In the final set of experiments, the potential interactions between central KiSS-1 and relevant neurotransmitters in the neuroendocrine control of LH secretion were explored. Thus, in experiment 8, the LH-releasing effect of central administration of KiSS-1 was monitored after blockade of NMDA and KA/AMPA receptors; *i.e.* the major ionotropic receptors for the excitatory amino acid (EAA) glutamate. To this end, groups of prepubertal (30 d old) male rats were ip treated with the NMDA receptor antagonist MK-801 (1 mg/kg) or the KA/AMPA receptor antagonist NBQX (0.5 mg/kg), in agreement with previous references (22, 23). Forty-five minutes after injection, 1 nmol KiSS-1 was icv injected, and trunk blood samples were taken on decapitation of the animals 15 min later. Similarly, NO dependency for the effects of KiSS-1 on LH secretion was assessed in experiment 9. Groups of prepubertal (30 d old) male rats were ip injected with the blocker of NO synthases NAME (40 mg/kg), as previously described (22). Forty-five minutes

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TABLE 1. Primer pairs used for RT-PCR amplification of LHRH and RP-S11 transcripts in hypothalamic samples

Target	Oligo-primers (5′–3′)	Expected size (bp)	PCR cycles
LHRH			
LHRH sense	GCA CTA TGG TCA CCA GCG GG	477	32
LHRH as	CAT GGA TCT CAG CGT CAA TG		
RP-S11			
S11 sense	CAT TCA GAC GGA GCG TGC TTA C	240	24
S11 as	TGC ATC TTC ATC TTC GTC AC		

The expected size of the generated cDNA products and the number of cycles selected for RT-PCR analysis is indicated for each signal.

after injection, 1 nmol KiSS-1 was icv injected, and trunk blood samples were taken on decapitation of the animals 15 min later.

Hormone measurement by specific RIAs

Serum LH levels were measured in a volume of 25 μl using a double-antibody method and RIA kits kindly supplied by the National Institutes of Health (Dr. A. F. Parlow, National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Peptide Program, Bethesda, MD). Rat LH-I-9 was labeled with ^{125}I by the chloramine-T method, and the hormone concentrations were expressed using the reference preparation LH-RP-3 as standards. Intra- and interassay coefficients of variation were less than 8 and 10%, respectively. The sensitivity of the assay was 5 pg/tube. In addition, in selected serum samples (experiment 1), serum testosterone levels were determined using a commercial kit from MP Biomedicals (Costa Mesa, CA), following the instructions of the manufacturer. The sensitivity of the assay was 0.1 ng/tube, and the intraassay coefficient of variation was less than 5%. Accuracy of hormone determinations was confirmed by assessment of rat serum samples of known hormone concentrations used as external controls.

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). Expression of LHRH mRNA was assessed by RT-PCR, optimized for semiquantitative detection, using the primer pairs and conditions indicated in Table 1. As internal control for reverse transcription (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 of total RNA was used to perform RT-PCR in two consecutive separate steps. In addition, to enable appropriate amplification in the exponential phase for each target, PCR amplification of LHRH and RP-S11 transcripts was 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 (16, 25). PCRs consisted in 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 at 58 C for 30 sec, and extension at 72 C for 1 min. A final extension cycle of 72 C for 15 min was included. Different numbers of cycles were tested to optimize amplification in the exponential phase of PCR. On this basis and our previously published work (16), the numbers of PCR cycles indicated in Table 1 were chosen for further semiquantitative analysis of LHRH 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. 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, Spain). Quantification of intensity of RT-PCR signals was carried out by densitometric scanning using an image analysis system (1-D Manager, TDI Ltd., Madrid, Spain), and values of the specific signal (LHRH) 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.

Presentation of data and statistics

Serum LH and testosterone determinations were conducted in duplicate, with a minimum total number of 10 samples/determinations per group. Hormonal data are presented as mean ± SEM. In addition, when appropriate (see experiment 6), integrated LH secretory responses were expressed as the area under the curve (AUC), calculated following the trapezoidal rule, over a 240-min period after iv administration of KiSS-1 peptide. Semiquantitative RT-PCR analyses were carried out in duplicate from at least four independent RNA samples of each experimental group. For generation of RNA samples, two hypothalamic fragments were pooled before isolation, and the generated samples were processed independently. Semiquantitative RNA data are presented as mean ± SEM. Results were analyzed for statistically significant differences using Student's *t* test or ANOVA followed by Student-Newman-Keuls multiple range test (SigmaStat 2.0, Jandel Corp., San Rafael, CA). $P \leq 0.05$ was considered significant.

Results

Dose-response analysis of KiSS-1 effect on LH secretion and its interaction with LHRH

The effects of central (icv) administration of a range of doses of KiSS-1, from 1 nmol to 10 pmol, on LH secretion were monitored in male rats, at 15 and 60 min after injection of the peptide. Notably, such an analysis was conducted in pubertal (45 d old) rats. This age point was selected, given the previously proposed key role of the KiSS-1/ GPR54 system in puberty onset (14). Moreover, the highest expression levels of GPR54 gene at the hypothalamus are detected in pubertal animals, thus suggesting maximum sensitivity of the system at this stage (16). Fifteen minutes after KiSS-1 administration, all doses tested (1 nmol and 500, 100, and 10 pmol) maximally elicited LH secretion (~7- to 8-fold increase over controls). In contrast, although 60 min after injection all doses of KiSS-1 significantly stimulated LH levels, the magnitude of elevation of serum LH levels at this time point appeared as dose dependent, with peak values at 1 nmol and 500 pmol, and lower responses at 100 and 10 pmol KiSS-1 (Fig. 1). In good

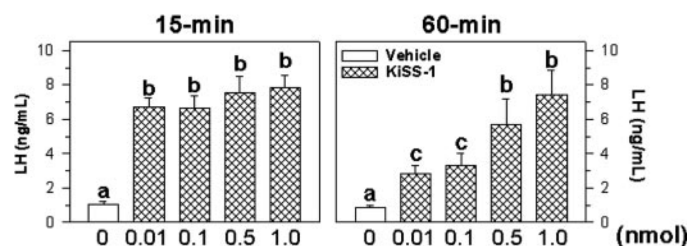

FIG. 1. Potent LH-releasing activity of centrally administered KiSS-1 peptide. A detailed dose-response analysis of the effects of icv administration of KiSS-1 on serum LH levels is presented. A range of doses of KiSS-1 (1, 0.5, 0.1, and 0.01 nmol) was tested for icv injection, and serum LH concentrations were determined 15 (*left panel*) and 60 min (*right panel*) after administration of the peptide. Values are the mean ± SEM of at least 10 independent determinations per group. Groups with *different superscript letters* are significantly different ($P < 0.01$, ANOVA followed by Student-Newman-Keuls multiple range test).

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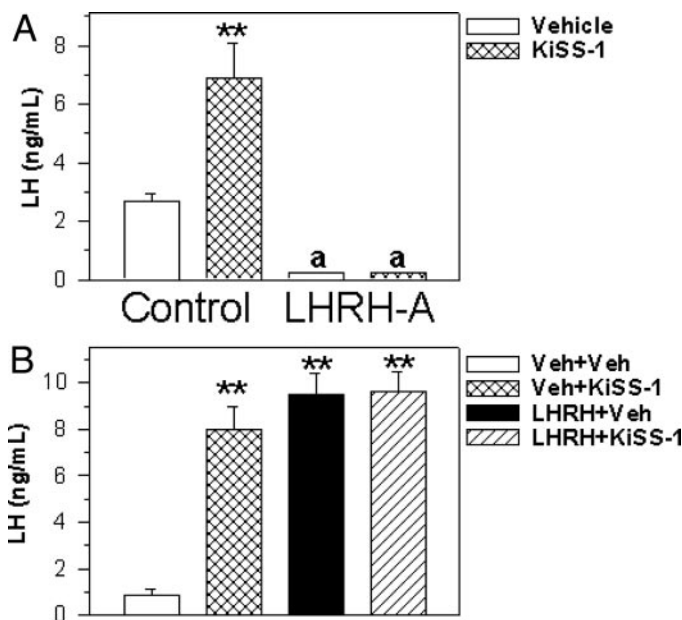

FIG. 2. Analysis of the interplay between KiSS-1 and hypothalamic LHRH in the central control of LH secretion. A (*upper panel*), Blockade of endogenous LHRH actions abolished the ability of KiSS-1 to stimulate LH secretion. Male rats were sc treated with a potent LHRH antagonist (LHRH-A; 5 mg/kg·24 h, two doses) or vehicle (control) and were subsequently icv injected with vehicle or 1 nmol KiSS-1. B (*lower panel*), The effects of coadministration of LHRH and KiSS-1 peptide on serum LH levels are shown. Male rats ip injected with 1 μg (0.85 nmol) LHRH or vehicle (veh) were simultaneously subjected to central (icv) administration of 1 nmol KiSS-1 or vehicle. Central administration of KiSS-1 failed to further elicit serum LH levels over LHRH-stimulated values. Hormonal data are the mean ± SEM of at least 10 independent determinations. **, $P < 0.01$ *vs.* corresponding vehicle control group; a, $P < 0.01$ *vs.* control KiSS-1-injected group in the *upper panel* (ANOVA followed by Student-Newman-Keuls multiple range test).

agreement with serum LH data, maximal responses to 1 nmol KiSS-1 at 60 min correlated with an extremely potent secretory effect in terms of testosterone release, whose serum levels were increased approximately 10-fold over control values (6.99 ± 0.85 *vs.* 0.75 ± 0.09 ng/ml in vehicle-injected animals).

Given the potent releasing effect of centrally administered KiSS-1 on LH secretion, its potential interaction with hypothalamic LHRH, *i.e.* the major direct elicitor of gonadotropin secretion (15, 26), was addressed *in vivo*. To this end, maximally effective doses (1 nmol/rat) of KiSS-1 were used. First, LHRH dependency for the stimulatory actions of KiSS-1 was assessed in pubertal males previously subjected to the blockade of the actions of endogenous LHRH by means of administration of a potent synthetic antagonist. To completely block LHRH actions without inducing a severe impairment of the secretory capacity of gonadotrope cells, a regimen of two injections of the antagonist at a 24-h interval was selected. Treatment with LHRH antagonist resulted in a significant drop in serum LH levels to nearly undetectable values, in line with previous reports (20). In this setting, central administration of 1 nmol KiSS-1 failed to induce a significant increase in serum LH levels in LHRH antagonist-treated animals, despite a potent LH-releasing activity of KiSS-1 confirmed in paired vehicle-injected animals (Fig. 2A). Second, the effects of systemic administration of LHRH were compared with those of central injection of KiSS-1. As shown in Fig. 2B, administration of 1 μg/rat of LHRH maximally elicited LH secretion, with approximately 8- to 9-fold increase over controls. This response was mimicked by icv injection of 1 nmol (equivalent to 1.3 μg) KiSS-1 (~8-fold increase over controls). Combined administration of LHRH and KiSS-1 was not able to further increase serum LH levels over those observed after individual administration of both peptides. Third, the effects of KiSS-1 on the expression levels of LHRH mRNA were monitored. Chronic central administration of KiSS-1 peptide (1 nmol per 12 h for 7 d) to pubertal males did not significantly modify relative expression levels of LHRH gene in whole hypothalamic/POA samples at 60 min after last injection (Fig. 3). Similarly, acute icv injection of 1 nmol KiSS-1 failed to induce significant changes in LHRH mRNA levels in whole hypothalamic/POA tissue over a period of 180 min (data not shown).

Effects of systemic administration of KiSS-1 on LH secretion and direct pituitary actions

In the next step, the effects of systemic injection of KiSS-1 on LH secretion were monitored using models of systemic (ip and iv) administration. Intraperitoneal injection of 7.5 nmol

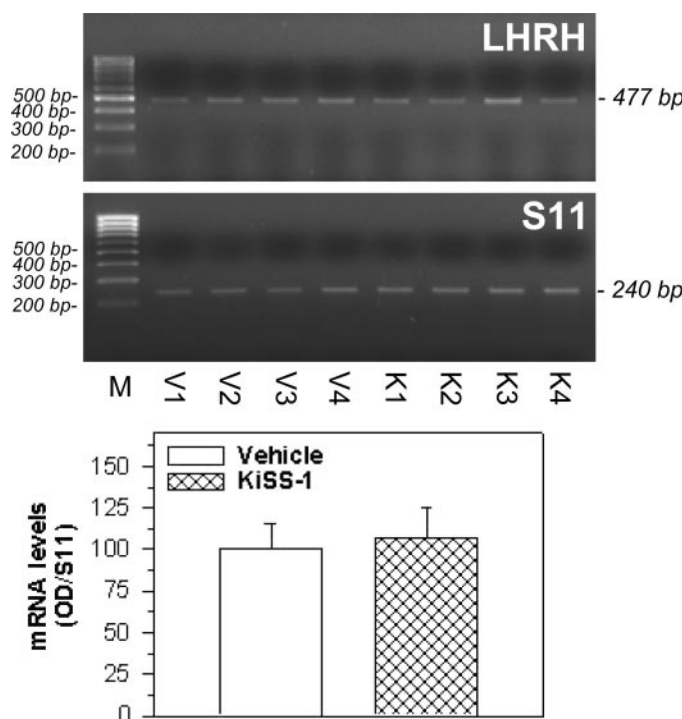

FIG. 3. Lack of effect of central administration of KiSS-1 peptide on relative LHRH mRNA levels in whole hypothalamic/POA tissue. In the *upper panel*, a representative RT-PCR assay of the expression levels of LHRH mRNA after chronic icv administration of 1 nmol KiSS-1 every 12 h for 7 d at 60 min after last injection is shown. Vehicle-injected animals served as controls. Four independent samples per group (vehicle: V1-V4; KiSS-1: K1-K4) are presented. Parallel amplification of S-11 ribosomal protein mRNA served as internal control. In the *lower panel*, semiquantitative values of LHRH mRNA levels are the mean ± SEM of duplicates from four independent determinations. No significant difference between groups injected with vehicle or KiSS-1 was detected (unpaired Student's t test).

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KiSS-1 was able to significantly elicit LH secretion to a similar extent as central administration of 1 nmol KiSS-1. Comparative time-course analysis of the responses to ip and icv treatments with KiSS-1 evidenced, however, that, although similar maximum responses were achieved at 15 min, the decay in serum LH levels after ip injection of KiSS-1 took place faster than after icv administration, and 60 min after ip delivery of KiSS-1, serum LH concentrations were virtually similar to those of vehicle-injected controls (Fig. 4). Likewise, iv administration of 7.5 nmol KiSS-1 elicited a potent LH secretory response in freely moving male rats. Thus, analysis of individual profiles of LH secretion demonstrated peak responses at 15 to 30 min after iv administration of KiSS-1 and a significant decline in circulating LH values thereafter. Accordingly, the integrated LH secretion during the 240-min period of sampling, as estimated by the AUC, was significantly increased by iv injection of 7.5 nmol KiSS-1 (Fig. 5).

Because results from experiments involving systemic administration did not exclude the possibility that, in addition to central actions, KiSS-1 may act directly at the pituitary level to modulate LH secretion, challenge of pituitary tissue with increasing doses of KiSS-1 peptide (10^{-10}, 10^{-8}, 10^{-6} M) was conducted using a static incubation system. Secretory responses were determined at 60 and 180 min of incubation with the stimuli. In this setting, KiSS-1 peptide was able to elicit LH secretion by incubated pituitary tissue in a dose-dependent manner; a significant minor effect was observed at 10^{-10} M KiSS-1, whereas 10^{-8} and 10^{-6} M doses further stimulated *in vitro* LH secretion (at 60- and 180-min periods). In terms of biopotency, the maximum releasing effect of 10^{-6} M KiSS-1 was significantly lower than that of 10^{-8} M LHRH, which was able to induce an *in vitro* secretory response approximately 4-fold higher than that of an equimolar dose of KiSS-1 (Fig. 6).

Analysis of interaction among KiSS-1, EAAs, and NO in the control of LH secretion

Finally, the potential interaction of KiSS-1 with other relevant signals in the central neuroendocrine control of LH

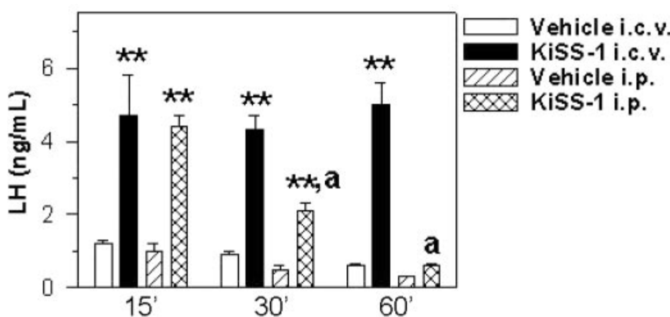

FIG. 4. Potent LH-releasing effect of KiSS-1 peptide after systemic administration. A comparative analysis of the effects of systemic (ip) or central (icv) administration of KiSS-1 peptide on LH secretion over a 60-min period is presented. Administration of KiSS-1 peptide (1 nmol per rat icv; 7.5 nmol per rat ip) was conducted in groups of pubertal animals, and serum LH concentrations were determined at 15, 30, and 60 min. Vehicle-injected (icv and ip) groups served as controls. Hormonal values are the mean ± SEM of at least 10 independent determinations. **, $P < 0.01$ *vs.* corresponding vehicle-injected groups; a, $P < 0.01$ *vs.* corresponding icv KiSS-1-injected group (ANOVA followed by Student-Newman-Keuls multiple range test).

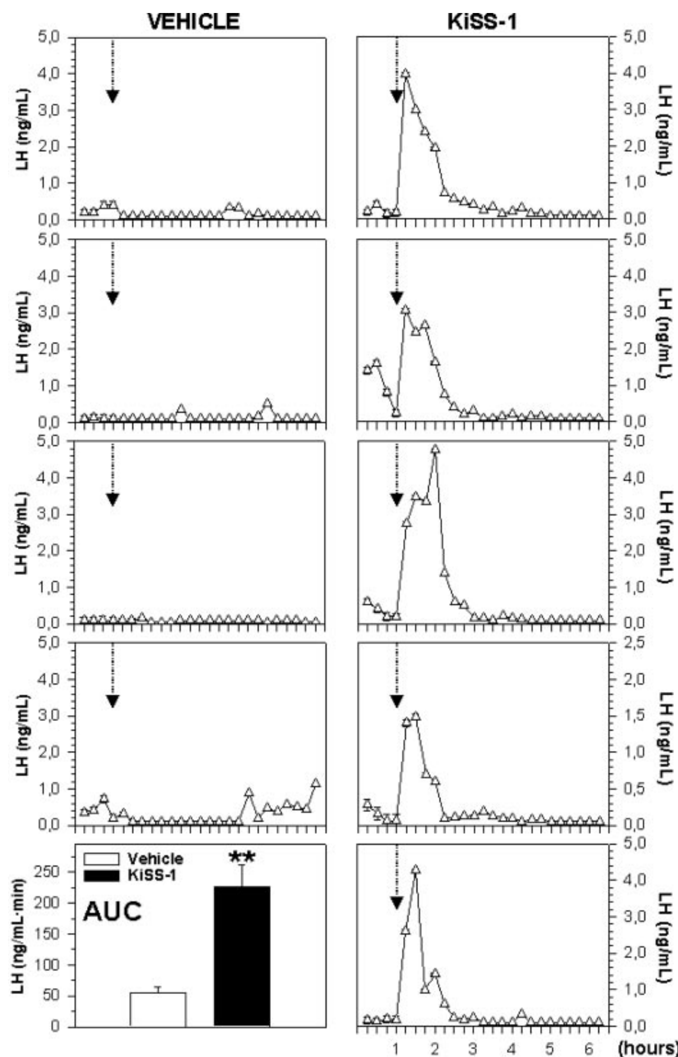

FIG. 5. Potent LH-releasing effect of KiSS-1 peptide after iv administration, analyzed in freely moving conditions. Individual serum LH profiles in male rats iv injected with vehicle or 7.5 nmol KiSS-1 are presented. Administration of the peptide or vehicle took place after stabilization of the animals after the fourth sampling point (*arrow*). In addition, the mean integrated LH secretory responses from vehicle- and KiSS-1-injected groups, calculated as the AUC over the study period (240 min), are shown. **, $P < 0.01$ *vs.* corresponding vehicle-injected group (unpaired Student's *t* test).

secretion was explored using *in vivo* models of pharmacological antagonization. Notably, the available data strongly suggested that the actions of KiSS-1 system in the control of the gonadotropic axis and LH secretion are mostly conducted at central levels (Refs. 14, 16, and 17; and present results). Thus, the ability of central (icv) injection of maximally effective doses of KiSS-1 to elicit LH secretion was monitored after antagonization of ionotropic glutamate receptors, of the NMDA and non-NMDA type, as well as after inhibition of endogenous NO synthases (NOS). In keeping with previous references on the prominent role of NMDA receptors in the control of LH secretion at puberty (27, 28), administration of the specific antagonist of NMDA receptors MK-801 induced a significant decrease in serum LH levels, which was not detected after injection of the blocker of KA/

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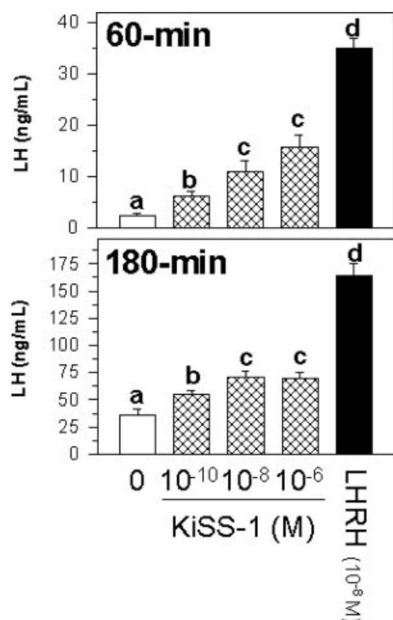

FIG. 6. Direct stimulatory effect of KiSS-1 peptide on LH secretion by incubated pituitary tissue. Pituitaries were challenged with increasing concentrations of KiSS-1 (10^{-10} to 10^{-6} M) or 10^{-8} M LHRH. Secretory responses at 60 and 180 min of incubation in the presence of the stimuli are shown. Hormonal values are the mean ± SEM of at least 10 independent determinations. Groups with *different superscript letters* are significantly different ($P < 0.01$, ANOVA followed by Student-Newman-Keuls multiple range test).

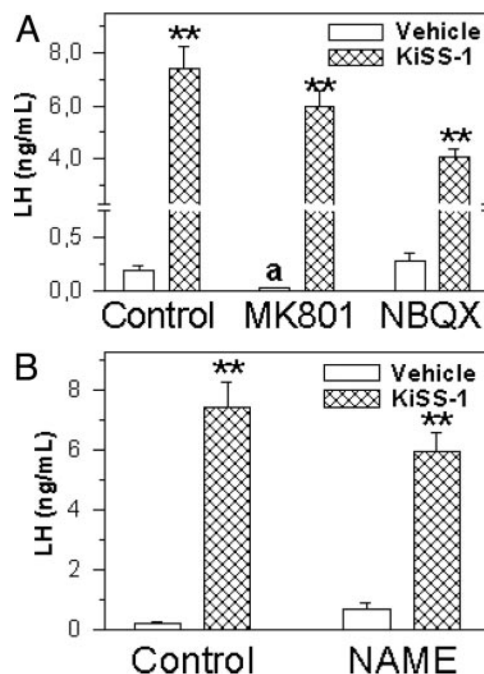

FIG. 7. Interplay between KiSS-1, EAA, and NO pathways in the central control of LH secretion. A (*upper panel*), Blockade of endogenous EAA pathways did not abolish the potent LH-releasing effect of KiSS-1 peptide. Prepubertal male rats were sc treated with the selective antagonists of NMDA and KA/AMPA receptors, MK-801 and NBQX, respectively, and were subsequently icv injected with 1 nmol KiSS-1 or vehicle. B (*lower panel*), Blockade of endogenous NO tone did not modify the ability of KiSS-1 peptide to stimulate LH secretion. Prepubertal male rats were sc treated with the inhibitor of NOSs NAME and were subsequently icv injected with 1 nmol KiSS-1 or vehicle. Hormonal values are the mean ± SEM of at least 10 independent determinations. **, $P < 0.01$ *vs.* corresponding icv vehicle-injected groups; a, $P < 0.01$ *vs.* corresponding control values in the *upper panel* (ANOVA followed by Student-Newman-Keuls multiple range test).

AMPA receptors, NBQX. In this setting, neither pretreatment with MK-801 nor administration of NBQX was able to significantly alter the LH-releasing effect of icv injection of 1 nmol KiSS-1 (Fig. 7A). Similarly, the potent LH secretory response to icv injection of KiSS-1 was persistently detected after pretreatment with the antagonist of NOSs NAME (Fig. 7B).

Discussion

Pituitary LH is a glycoprotein hormone with key roles in the control of pivotal gonadal functions such as ovulation, spermatogenesis, and sex hormone production (15). The neuroendocrine control of LH secretion is driven by a complex network integrating central and peripheral signals, in which the hypothalamic decapeptide LHRH is the final regulatory factor that ultimately dictates the pulsatile release of LH (as well as FSH) from pituitary gonadotropes (15, 26). In turn, episodic release of LHRH is exquisitely governed by the interplay of a plethora of excitatory and inhibitory signals at the hypothalamus (26). Although the nature of such signals has been partially elucidated over the last decades, it is likely that additional, as-yet-unknown central neurotransmitters may participate in the control of the LHRH-LH system. Recently the KiSS-1/GPR54 system has been pointed out as a relevant central regulator of the gonadotropic axis (13, 14). Indeed, hypothalamic expression of KiSS-1 and GPR54 genes has been proven as developmentally (maximum at puberty) and hormonally (by sex steroids) regulated (16). Moreover, expression of KiSS-1 gene has been recently demonstrated in key hypothalamic areas implicated in the neuroendocrine control of gonadotropin secretion (17).

In this context, our current data document the extraordinarily potent LH-releasing activity of centrally administered KiSS-1 peptide, in keeping with our initial observations on the effects of icv injection of 1 nmol KiSS-1 (16). Thus, dose-response analyses evidenced that an input as low as 10 pmol KiSS-1 was able to maximally activate LH secretion to a level similar to that induced by systemic administration of LHRH, 15 min after injection. Although a certain degree of dose dependency was detected for the decay of the effects of KiSS-1 on serum LH levels over a 60-min period, it becomes evident by comparison with previously published data on the LH-releasing activity of other neuropeptides and neurotransmitters, such as glutamate and galanin-like peptide (28, 29), that the biopotency of KiSS-1 is considerably higher than that of other known regulators of LH secretion. In fact, during the final stage of preparation of this manuscript, Gottsch *et al.* (17) independently reported a detailed dose-response analysis of the effect of a wide range of doses of KiSS-1 peptide (1 fmol to 5 nmol) on LH secretion in the mouse. Although a single (30 min) time point was screened at that study, those data fully support our present observations and further extend the characterization of the extraordinarily potent LH-releasing activity of KiSS-1 peptide be-

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cause a dose as low as 1 fmol of kisspeptin-54 was able to significantly increase serum LH levels (17).

The mechanisms whereby central administration of KiSS-1 peptide maximally activates LH secretion were addressed by a combination of approaches, targeting the putative contribution of hypothalamic LHRH to such a phenomenon. Our results demonstrate that, despite the use of maximally effective doses of KiSS-1 peptide, its ability to centrally elicit LH secretion is totally abrogated in the absence of endogenous LHRH actions in the male, in keeping with our recent observations in immature female rats (Navarro, V. M., and M. Tena-Sempere, submitted material). In addition, icv injection of KiSS-1 equaled the maximum stimulation of LH secretion induced by systemic administration of LHRH, but such a response could not be further increased by the combined administration of LHRH (ip) and KiSS-1 (icv). Although we cannot rule out the possibility that maximum LH responses were obtained at the doses used for each individual peptide, such a lack of additivity indirectly points out the convergence of KiSS-1 and LHRH pathways in a level upstream of the LH-secreting cells. Overall, our data strongly suggest that the effects of central KiSS-1 on LH secretion are mainly conducted through modulation of LHRH system. In this sense, GPR54 gene expression has been very recently demonstrated in LHRH neurons from the nonmammalian species tilapia (*Oreochromis niloticus;* cichlid fish) (30). Similarly, our preliminary data evidence that GPR54 gene is also expressed in the murine LHRH neuronal cell line GT1–7 (Magni, P., and M. Tena-Sempere, manuscript in preparation). Thus, it is tempting to propose that KiSS-1 peptide, acting through its receptor GPR54, is able to directly elicit the secretory activity of hypothalamic LHRH neurons. Although the precise mechanisms whereby this action is eventually conducted are yet to be identified, it appears likely that the potent LH-releasing effect of KiSS-1 is not due to the transcriptional activation of LHRH gene, as evidenced by our expression data of LHRH mRNA levels after icv injection of highly effective doses of KiSS-1. Nevertheless, the possibility of nucleus-specific effects of KiSS-1 on LHRH gene expression in a subset of LHRH neurons cannot be excluded on the basis of our current data. Alternatively, KiSS-1 might selectively stimulate LHRH release, in keeping with indirect evidence obtained in mouse models bearing mutations of GPR54 gene (14). This phenomenon is presently under evaluation at our laboratory.

Despite solid evidence pointing to a central hypothalamic system involving KiSS-1 and LHRH in the control of LH secretion, not only intracerebroventricular but also systemic (ip and iv) administration of KiSS-1 was able to significantly activate LH secretion. In fact, the effect of peripheral ip administration of KiSS-1 was similar in terms of maximum mean response to that of central injection of the peptide. Moreover, iv injection of KiSS-1 to freely moving animals sharply elicited a massive LH pulse in every single animal tested (see Fig. 5). Several mechanisms may account for such a phenomenon. First, it is likely that systemically delivered KiSS-1 may regulate LHRH release by LHRH neuron nerve terminals located at the median eminence-arcuate nucleus complex, which is placed outside the blood-brain barrier (31). In this sense, expression of KiSS-1 gene has been very recently demonstrated in several hypothalamic areas, including the arcuate nucleus (17), thus evidencing the potential interaction between KiSS-1 and LHRH neurons at this location. In addition, however, it is possible that part of the stimulatory action of systemic KiSS-1 may derive from a direct releasing effect at the pituitary level because dose-dependent stimulation of LH secretion by incubated pituitary tissue was observed after challenge with increasing concentrations of KiSS-1. This would imply that, regardless of its predominant central effect in the control of the LHRH-LH axis, hypothalamic KiSS-1 might also operate as true hypophysiotropic factor involved in the control of LH secretion at the pituitary level. Notably, expression of GPR54 gene has been demonstrated at the pituitary (3); yet its pattern of cellular distribution and regulation by hormonal signals and at different stages of development remain to be elucidated in this tissue.

The relative contribution of direct pituitary effects to the potent releasing activity of KiSS-1 is likely minor (equimolar doses of KiSS-1 were 4 times less potent than 10^{-8} M LHRH *in vitro*, whereas systemic administration of KiSS-1 was as effective as LHRH *in vivo*) and would require the permissive presence of LHRH. Yet KiSS-1 was able to moderately elicit LH secretion *in vitro* at dose as low as 10^{-10} M, *i.e.* in a range that is close to the threshold levels for LHRH-induced LH stimulation (our personal observation); a phenomenon whose relevance merits further investigation. Nevertheless, from a functional standpoint, our current results document the potent LH-releasing ability of systemically delivered KiSS-1 peptides, which may have both physiological and therapeutical implications. On the former, it is noticeable that KiSS-1 peptides, such as metastin, are detected in plasma, and their concentrations dramatically increase under certain physiological conditions, such as pregnancy (12).

Considering that our experimental data suggested a predominant role of central KiSS-1/LHRH network in the control of LH secretion, we aimed at evaluating the potential interplay between KiSS-1 and other relevant neurotransmitters previously implicated in the neuronedocrine modulation of LH release, such as EAAs (glutamate) and NO (27, 32). In this sense, a prevalent role of hypothalamic glutamatergic pathways in the pubertal activation of the LHRH-LH axis has been demonstrated (27, 28), and a role for NO as a synchronizing factor for the disperse population of LHRH neurons has been proposed (32). Our results using *in vivo* models of pharmacological blockade of ionotropic EAA receptors of the NMDA and non-NMDA type as well as inhibition of NOSs in prepubertal male rats demonstrate that the integrity of EAA and NO neurotransmission is not needed for the expression of the potent LH-releasing ability of centrally administered KiSS-1. These observations suggest that KiSS-1 is placed in a step distal to (or eventually independent of) glutamate and NO actions within the central circuitry governing LHRH release. An interesting possibility is that those neurotransmitters might regulate the secretory activity of LHRH neurons by primary modulating the central expression of KiSS-1. Such a possibility is currently under analysis at our laboratory.

In summary, our results provide conclusive evidence for a potent stimulatory effect of KiSS-1 peptide on LH secretion.

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Such a releasing action is mostly conducted centrally, likely through modulation of the LHRH system. However, the contribution of stimulatory effects of KiSS-1 on LH secretion directly at the pituitary level cannot be excluded. Overall, the present results, in conjunction with our recent data on the developmental and hormonally regulated pattern of expression of KiSS-1 and GPR54 genes in the hypothalamus (16), and their potential involvement in the activation of the reproductive axis at puberty (Navarro, V. M., and M. Tena-Sempere, submitted material), further document a novel role of the KiSS-1/GPR54 system as a relevant downstream element in the neuroendocrine network governing LH secretion.

Acknowledgments

RIA kits for hormone determinations were kindly supplied by Dr. A. F. Parlow (National Institute of Diabetes and Digestive and Kidney Disorders, National Hormone and Peptide Program).

Received July 1, 2004. Accepted September 9, 2004.

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This work was supported by Grants BFI 2000-0419-CO3-03 and BFI 2002-00176 from DGESIC (Ministerio de Ciencia y Tecnología, Spain), and European Union research contract EDEN QLK4-CT-2002-00603.

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