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A Role for Kisspeptins in the Regulation of Gonadotropin Secretion in the Mouse

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Kisspeptins are products of the KiSS-1 gene, which bind to a G protein-coupled receptor known as GPR54. Mutations or targeted disruptions in the GPR54 gene cause hypogonadotropic hypogonadism in humans and mice, suggesting that kisspeptin signaling may be important for the regulation of gonadotropin secretion. To examine the effects of kisspeptin-54 (metastin) and kisspeptin-10 (the biologically active C-terminal decapeptide) on gonadotropin secretion in the mouse, we administered the kisspeptins directly into the lateral cerebral ventricle of the brain and demonstrated that both peptides stimulate LH secretion. Further characterization of kisspeptin-54 demonstrated that it stimulated both LH and FSH secretion, at doses as low as 1 fmol; moreover, this effect was shown to be blocked by pretreatment with acyline, a potent GnRH antagonist. To learn more about the functional anatomy of kisspeptins, we mapped the distribution of KiSS-1 mRNA in the hypothalamus. We observed that KiSS-1 mRNA is expressed in areas of the hypothalamus implicated in the neuroendocrine regulation of gonadotropin secretion, including the anteroventral periventricular nucleus, the periventricular nucleus, and the arcuate nucleus. We conclude that kisspeptin-GPR54 signaling may be part of the hypothalamic circuitry that governs the hypothalamic secretion of GnRH. (*Endocrinology* 145: 4073–4077, 2004)

IN 1999, LEE AND COLLEAGUES (1) discovered a novel G protein-coupled receptor in the rat termed GPR54. The GPR54 gene encodes a G protein-coupled receptor that shares a modest sequence homology with two of the known galanin receptors (1); however, galanin does not bind to GPR54 *in vitro* (1). Instead, GPR54 has been shown to mediate the actions of a unique family of ligands known as kisspeptins, which are derived from the KiSS-1 gene. The KiSS-1 gene encodes a 145-amino acid peptide that is cleaved into an amidated C-terminal 54 amino acid product, known as either kisspeptin-54 or metastin, which is thought to be an endogenous ligand for GPR54 (2–4). Shorter fragments of kisspeptin-54 (*e.g.* kisspeptin-14, kisspeptin-13, and kisspeptin-10) also bind to GPR54 (2–4). Kisspeptin-54 was characterized, and named metastin, based on its ability to hinder tumor metastasis (2–5), but recent evidence suggests that kisspeptins and GPR54 may also play an important role in the neuroendocrine regulation of reproduction.

Signaling through GPR54 is essential for pubertal maturation of the reproductive system. Humans and mice having either dysfunctional or deletional mutations in GPR54 fail to undergo normal pubertal development as a result of hypogonadotropic hypogonadism (6–8). Although the precise anatomical distribution of KiSS-1 and GPR54 are not well characterized, both are expressed in the mammalian forebrain (1, 9), and thus, we postulated that kisspeptin/GPR54 signaling in the hypothalamus may be involved in the neuroendocrine regulation of gonadotropin secretion. We had three specific objectives. The first was to examine the effects of centrally administered kisspeptin-54 (metastin) and kisspeptin-10 on LH secretion and to create a full dose-response curve for kisspeptin-54. Second, having observed that kisspeptin-54 stimulated gonadotropin secretion, we tested the hypothesis that the kisspeptin-54-induced gonadotropin response (LH and FSH) was mediated by GnRH. We accomplished this by examining whether pretreatment with acyline, a potent GnRH antagonist, could block the kisspeptin-54-induced release of LH and FSH. The third objective was to map the detailed distribution of KiSS-1 mRNA within the hypothalamus of the mouse, searching for its presence in areas known to be involved in the regulation of GnRH secretion.

Materials and Methods

Animals and chemicals

Adult male C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were individually housed and were maintained on a 12-h light, 12-h dark cycle (lights on at 0600 h). All animals had access to standard rodent chow and water *ad libitum*. All procedures were approved by the Animal Care Committee of the School of Medicine of the University of Washington in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Kisspeptin-54 [KiSS-1 (68–119)-NH2 (mouse)/metastin (1–52)] was purchased from Phoenix Pharmaceutical (Belmont, CA). Kisspeptin-10 [KiSS-1 (112–121)/ metastin (45–54) (human)] was provided by the Peptide Core Facility of the Massachusetts General Hospital (Boston, MA). Acyline, a GnRH antagonist (10–12), was a gift from Dr. William J. Bremner (University of Washington, Seattle, WA). Acyline was synthesized by Jean Rivier at The Salk Institute (La Jolla, CA) and distributed by the National Institute of Child Health and Human Development (10).

Abbreviations: aCSF, Artificial cerebrospinal fluid; ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus; DMSO, dimethylsulfoxide; GPR54, a novel G protein-coupled receptor; ICV, intracerebroventricular; PeN, periventricular nucleus.

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Intracerebroventricular (ICV) injections

Freehand ICV injections in the lateral cerebral ventricle were performed as previously described (13, 14). Briefly, mice were anesthetized with isoflurane (Abbott Laboratory, North Chicago, IL) delivered by a vaporizer (Veterinary Anesthesia Systems, Bend, OR). Upon achieving a surgical plane of anesthesia, a small hole was bored in the skull 1 mm lateral and 0.5 mm posterior to bregma with a Hamilton syringe attached to a 27-gauge needle fitted with polyethylene tubing, leaving 3.5 mm of the needle tip exposed. Once the initial hole was made, all subsequent injections were made at the same site. Mice were allowed to recover for at least 2 d before treatment. For ICV injections, mice were anesthetized with isoflurane for a total of 2–3 min, during which time 3 μl of solution were slowly and continuously injected into the lateral ventricle. The needle remained inserted for approximately 60 sec after the injection to minimize backflow up the needle track. Mice typically recovered from the anesthesia within 3 min after the injection.

RIAs

Serum LH and FSH concentrations were measured with reagents obtained from the National Institutes of Health. For LH, the antiserum used was anti-rLH-S-11, and the standard was rLH-RP3. The assay sensitivity was 0.2 ng/ml, and the intraassay coefficient of variation was 7%. For FSH, the antiserum used was anti-rFSH-S-11, and the standard was rFSH-RP2. The assay sensitivity was 1.0 ng/ml, and the intraassay coefficient of variation was 6%. Both assays have been validated for use in the mouse.

Experiment 1: LH response to ICV kisspeptin-54 and kisspeptin-10

Mice were handled daily for 2 wk before the experiment. Mice were given an ICV injection of one of four treatments: kisspeptin-54 (1 nmol) dissolved in artificial cerebrospinal fluid (aCSF), kisspeptin-10 (1 nmol) dissolved aCSF + 15% dimethylsulfoxide (DMSO), aCSF + 15% DMSO alone, or aCSF alone (n = 5 per group). Blood was obtained via orbital bleed 30 min post injection while mice were under isoflurane anesthesia, and sera were assayed for LH. We measured the LH response to both kisspeptin-54 and kisspeptin-10 to confirm the putative biological activity of both ligands in the context of gonadotropin secretion. The 30-min time interval between the injection and the blood sampling was selected based on the observation that other secretagogs that stimulate LH secretion in a GnRH-dependent fashion do so within this time frame (14, 15).

Experiment 2: LH response to variable doses of central kisspeptin-54

Mice were given an ICV injection of kisspeptin-54 in doses varying from 1 fmol to 5 nmol or aCSF alone (n = 5–8 per group). Doses administered include 1 fmol, 10 fmol, 0.1 pmol, 1 pmol, 10 pmol, 0.1 nmol, 0.375 nmol, 0.625 nmol, 1.25 nmol, 2.5 nmol, and 5 nmol. Blood was obtained via orbital bleed 30 min post injection while mice were under isoflurane anesthesia, and sera were assayed for LH.

Experiment 3: role of GnRH in mediating the effects of kisspeptin-54 on LH and FSH secretion

Mice received an sc injection of acyline (50 μg) dissolved in sterile saline (100 μl/mouse) 24 h and 1 h before ICV injection of kisspeptin-54 or aCSF alone. Control mice were given an sc injection of saline only at 24 h and 1 h before ICV injections. On the day of the experiment, mice received either an ICV injection of kisspeptin-54 (0.05 nmol) or aCSF alone. Of the mice that had been given acyline, half were treated with kisspeptin-54 and the other half received aCSF (n = 6/group). Similarly, half of the mice that had been treated with saline were given kisspeptin-54, and the other half received aCSF (n = 6/group). Blood was obtained by orbital bleed 60 min post ICV injection, while mice were under isoflurane anesthesia and sera were assayed for LH and FSH. Mice were not used in a crossover design.

Measurement of gonadotropins in the acyline treatment experiment was done at 60 min after the injection, upon reflection of the results from the first experiment, which had revealed that kisspeptin-54 caused a slight but not significant increase in serum levels of FSH (data not shown). Because the FSH response to secretagogs for gonadotropins is often delayed relative to LH, we decided to increase the interval between the injection and the blood sample by another 30 min to optimize the chance of revealing an FSH response to kisspeptin-54.

Experiment 4: distribution of KiSS-1 mRNA in the hypothalamus of the mouse

Tissue preparation. Mice were anesthetized with isoflurane and then killed by decapitation. Brains were removed and frozen on dry ice. Sections in the coronal plane (20 μm) were cut on a cryostat, thaw-mounted onto SuperFrost Plus slides (VWR Scientific, West Chester, PA), and stored at −80 C. Sections were collected from the diagonal band of Broca to the mammillary bodies.

Cloning of partial cDNA for mouse KiSS-1. Total RNA was extracted from mouse brain using an RNAqueous Kit (Ambion, Inc., Austin, TX). RNA was reverse transcribed into cDNA with a RetroScript kit (Ambion) primed with oligodeoxythymidine for subsequent PCR. Primers were designed based on the published sequence of the KiSS-1 mouse gene (GenBank accession no. AF472576) with forward primers corresponding to bases 76–93 and reverse primers corresponding to bases 466–486. Primers were custom synthesized (QIAGEN, Valencia, CA). PCRs contained the following in a volume of 25 μl: 2 μl of reverse transcriptase reaction product; 0.2 μM of each primer; 12.5 μl RediTaq polymerase (Sigma-Aldrich, Natick, MA); and 8.5 μl of water. Reactions were performed in a PTC-100 thermal cycler (MJ Research, Inc., Watertown, MA) using the following protocol: cDNA was denatured for 2 min at 94 C, then 35 cycles were carried out at 94 C for 1 min, 55 C for 1.5 min, and 72 C for 2 min, with a final 5 min extension at 72 C. After electrophoresis on a 2% agarose (wt/vol) gel, a single DNA fragment was obtained of approximately the expected size (411 bp) and gel purified with a QiaQuick gel extraction kit (QIAGEN). The PCR product was confirmed to be the mouse KiSS-1 probe by sequencing and was cloned into the pAMP1 plasmid (Invitrogen Life Technologies, Carlsbad, CA).

In situ hybridization. Antisense and sense mouse KiSS-1 probes were transcribed from linearized pAMP1 plasmid containing the mouse KiSS-1 insert with T7 and SP6 Polymerase Plus (Ambion), respectively. Radiolabeled probes were synthesized *in vitro* by inclusion of the following ingredients in a volume of 20 μl: 250 μCi ^{33}P-UTP (PerkinElmer Life Sciences, Boston, MA); 1 μg linearized DNA; 0.5 mM each ATP, CTP, GTP; 40 U polymerase. Residual DNA was digested with 4 U deoxyribonuclease (Ambion), and the deoxyribonuclease reaction was terminated by addition of 2 μl of 0.5 M EDTA (pH 8.0). The riboprobes were separated from unincorporated nucleotides with NucAway Spin Columns (Ambion).

Slides with mouse hypothalamic sections from three adult male C57BL/6 mice were processed before hybridization as previously reported (16). Radiolabeled antisense and sense KiSS-1 riboprobes were denatured, dissolved in hybridization solution at a concentration of 0.1 pmol/ml along with tRNA (1.9 mg/ml), and applied to slides. Two negative controls were used to demonstrate specificity of the KiSS-1 riboprobe: slides were incubated with radiolabeled antisense probe in the presence of excess (500×) unlabeled antisense probe, or with an equivalent concentration of radiolabeled sense KiSS-1 probe. Slides were covered with glass coverslips, placed in a humid chamber, and incubated overnight at 55 C. The next day, slides were washed as previously reported (16). Slides were then dipped in NTB-3 liquid emulsion (Eastman Kodak Co., Rochester, NY). Slides were developed 3 d later, and coverslips were applied.

Statistical analysis

All data are expressed as a mean ± SEM for each group. Differences among groups were assessed by one- or two-way ANOVA. When the ANOVA indicated significant differences, Fisher's *post hoc* test was used to identify differences between individual treatment groups. Student's *t* test was used when only two groups were being compared. Differences were considered significant when $P < 0.05$.

Results

Kisspeptin-54 (metastin) and kisspeptin-10 stimulate LH secretion in the mouse

The results demonstrate that kisspeptin-54 and kisspeptin-10 stimulate LH release in adult male mice, when kisspeptin-54 or kisspeptin-10 is administered via an ICV injection. Kisspeptin-54 and kisspeptin-10 stimulated LH secretion in the mouse ($P = 0.01$ *vs.* vehicle-treated animals) (Fig. 1). LH was not different in those injected with aCSF or aCSF + 15% DMSO (data not shown).

We determined the lowest effective dose of kisspeptin-54 that stimulates LH secretion by administering varying doses (1 fmol to 5 nmol) and measuring their effect on serum levels of LH. Kisspeptin-54 produced a significant increase in serum LH at all doses tested (Fig. 2). The responses to doses greater than 1 fmol were not significantly different from each other. The response to 1 fmol was intermediate—significantly greater than vehicle ($P < 0.05$) but less than the 10 fmol and higher doses ($P < 0.001$).

Kisspeptin-54 stimulates gonadotropin secretion by acting on the hypothalamus

To determine whether kisspeptin's stimulatory effect on gonadotropin secretion was mediated by GnRH, we pretreated animals with acyline, a potent GnRH antagonist, before delivering the ICV injection of kisspeptin-54. Kisspeptin-54 (0.05 nmol) significantly stimulated both LH ($P < 0.0001$) and FSH secretion ($P < 0.001$) compared with vehicle-treated animals. Gonadotropin responses to kisspeptin-54 were blocked in mice pretreated with acyline (kisspeptin-54 *vs.* vehicle: $P > 0.05$) (Fig. 3).

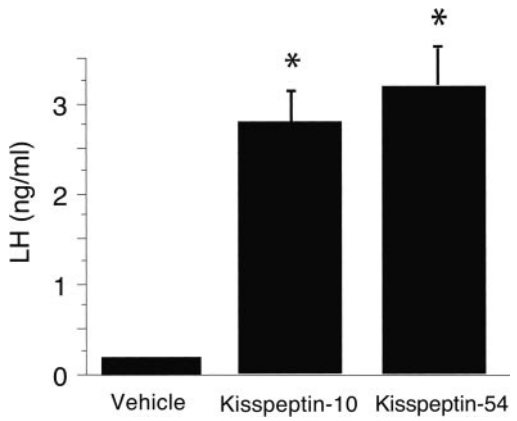

FIG. 1. Effects of kisspeptin-10 and kisspeptin-54 (1 nmol, delivered ICV) on serum levels of LH, measured at 30 min after a bolus injection. *, $P < 0.01$ *vs.* vehicle alone.

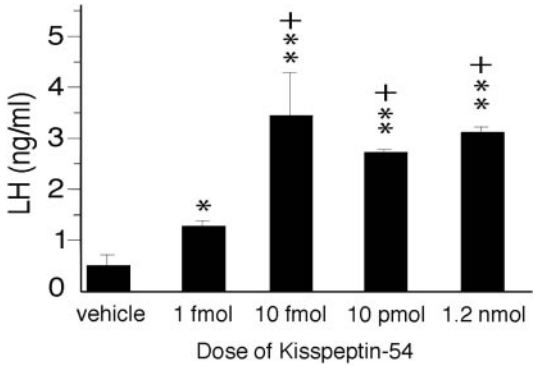

FIG. 2. Effects of different doses of kisspeptin-54 (ranging from 0–5 nmol delivered ICV) on serum levels of LH, measured 30 min after a bolus injection. *, $P < 0.01$ *vs.* vehicle; **, $P < 0.001$ *vs.* vehicle; +, $P < 0.001$ *vs.* 0.001 pmol kisspeptin-54.

Expression of KiSS-1 mRNA in the brain of the mouse

Silver grain clusters, representing cells expressing KiSS-1 mRNA, were found at several levels through the rostral-caudal extent of the hypothalamus. Many cells expressing KiSS-1 mRNA were observed in the anteroventral periventricular nucleus (AVPV), the periventricular nucleus (PeN), and the arcuate nucleus (ARC) (Fig. 4). In the rostral aspect of the ARC, KiSS-1 mRNA was found throughout both the medial and lateral divisions. However, in the caudal aspect of the ARC, KiSS-1 mRNA expression was restricted to the ventral portion of the nucleus. Some cells expressing KiSS-1 mRNA were observed in the anterodorsal preoptic nucleus, whereas few cells were found in the medial amygdala and bed nucleus of the stria terminalis. The amount of KiSS-1 mRNA per cell, as estimated by the number of silver grains per cluster, did not appear to differ significantly among these anatomical regions of the hypothalamus. Including excess unlabeled antisense probe with radiolabeled antisense probe abolished all specific signal, and no signal was observed after the application of radiolabeled sense probe (data not shown).

Discussion

We have shown that kisspeptin-54 delivered directly into the lateral cerebral ventricle stimulates LH and FSH secretion. Kisspeptin-54 is remarkable in that it elicits gonadotropin secretion at doses as low as 1 fmol. There are no directly comparable studies showing full dose-response curves for other neuropeptides and their effect on LH secretion in the adult male mouse; however, based on studies of single effective doses of other neuropeptides, such as neuropeptide Y (15, 17), galanin (15), and galanin-like peptide (14, 18), and excitatory amino acids such as glutamate and *N*-methyl-D-aspartate (19), in a variety of rodent species, it would appear that kisspeptin-54 is several orders of magnitude more potent than any of these other secretagogs for LH.

Our results also demonstrate that the ability of kisspeptin-54 to stimulate gonadotropin release is dependent upon GnRH secretion—based on the observations that pretreatment with a GnRH antagonist blocks the effect of kisspeptin-54. These results are consistent with the phenotype of GPR54-deficient mice whose pituitaries are capable of secreting LH in response to exogenous GnRH, despite their gonadotropin deficiency. Together, these observations argue that the hypogonadism in humans and mice with dysfunctional or deletional mutations in GPR54 is attributable to a central defect (7). Based on these findings, we infer that kisspeptin-54 activates its cognate receptor GPR54 to provide an obligatory activational signal to GnRH neurons at the time of puberty. However, precisely where in the brain kisspeptin-54 acts to stimulate GnRH secretion is unknown. Although GPR54 mRNA is expressed throughout the forebrain (1, 9)—notably

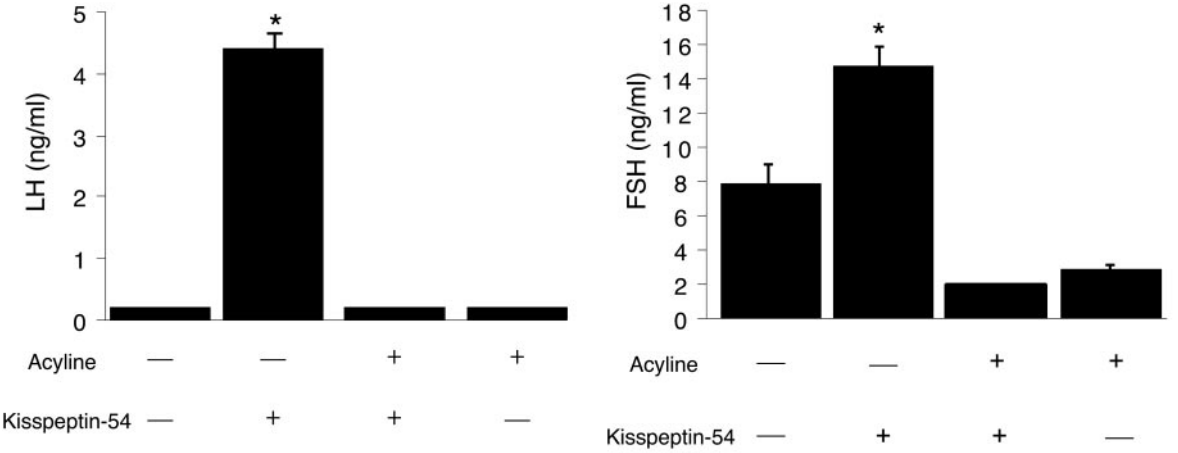

FIG. 3. The effects of kisspeptin-54 (50 pmol delivered ICV) or its vehicle alone, coupled with pretreatment with a GnRH antagonist, acyline (50 μg, sc), or its vehicle alone. A, LH data; B, FSH data. *, $P < 0.001$ saline/kisspeptin-54 *vs.* all other treatments.

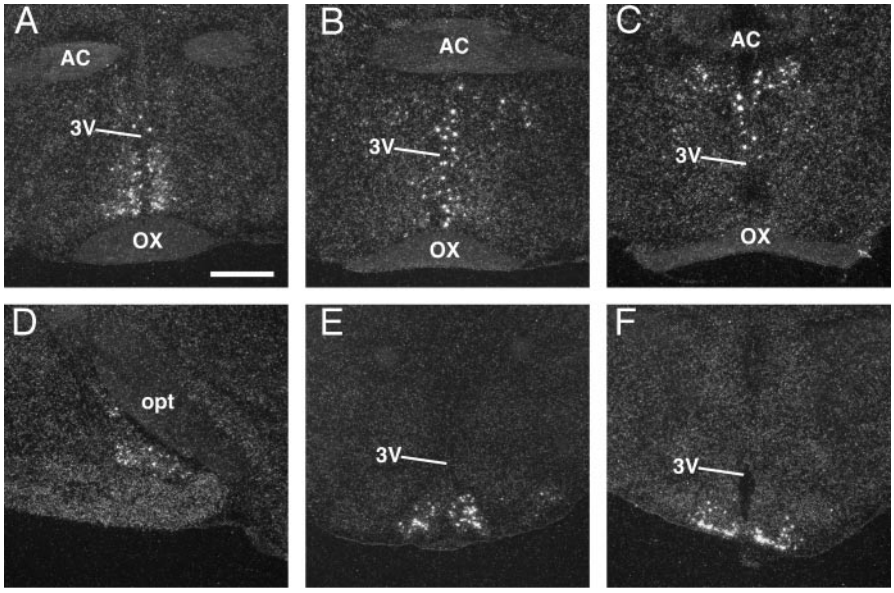

FIG. 4. Distribution of KiSS-1 mRNA expressing cells in the hypothalamus. *Clusters of white dots*, corresponding to clusters of silver grains where the labeled RNA probe has been concentrated, indicate KiSS-1 mRNA-expressing cells. KiSS-1 mRNA-containing cells were observed in the AVPV (A), the PeN (B), anterodorsal preoptic nucleus (C), the medial amygdala (D), and the ARC (E and F). 3V, Third ventricle; AC, anterior commissure; opt, optic tract; OX, optic chiasm. *Scale bar*, 500 μm.

in areas of the brain where GnRH neurons are known to reside (20)—it remains to be determined whether GnRH neurons themselves express GPR54 or the action of kisspeptin-54 on GnRH secretion is mediated indirectly by intervening neurons.

Despite the inference that kisspeptin-54 stimulates GnRH secretion, we cannot exclude the possibility that the centrally administered kisspeptin-54 gained access to the pituitary and thus directly stimulated LH and FSH release from the pituitary. This is at least conceivable because GPR54 is expressed in the pituitary (2, 9). However, we believe this explanation is improbable for two reasons. First, kisspeptin-54 is unusually potent at stimulating LH release, retaining its effectiveness at doses as low as 1 fmol. The efficacy of such low doses of kisspeptin-54 delivered into the brain to stimulate LH secretion would mitigate the probability that kisspeptin-54, diffusing and diluting from its site of delivery in the lateral ventricle could retain sufficient activity to stimulate pituitary gonadotropes. Second, the ability of kisspeptin-54 to stimulate LH secretion by a direct action on the pituitary should not have been impaired by the presence of the GnRH antagonist acyline. Nevertheless, it is still plausible that kisspeptin-54 could be acting synergistically with GnRH, similar to neuropeptide Y to affect LH release (21); this possibility cannot be excluded.

The distribution of KiSS-1 mRNA has been examined grossly in the brain of both the rat and human by RT-PCR, and transcripts for this gene are detectable (1, 9); however, the precise regional distribution of KiSS-1 mRNA within the forebrain has not been carefully annotated for any species. In this study in the mouse, we observed KiSS-1 mRNA-expressing cells in discrete hypothalamic nuclei, including the AVPV, PeN, and ARC. Muir and colleagues (9) also report finding significant expression of KiSS-1 in several other areas in the brain of the human—such as the caudate nucleus, globus pallidus, nucleus accumbens, putamen, and striatum; however, in the mouse, we did not find KiSS-1 mRNA in any

of these extrahypothalamic regions. Whether these apparent differences between the human and mouse represent real species differences or a disparity of the sensitivity in the respective techniques remains to be determined.

Based on the distribution of KiSS-1 mRNA, we surmise that kisspeptin-54 may be involved in the hypothalamic regulation of GnRH/gonadotropin secretion by gonadal steroids. Receptors for androgens, estrogens, and progesterone are expressed in the hypothalamic nuclei that express KiSS-1 mRNA, including the ARC and AVPV (22–24). These nuclei are both implicated in the negative feedback control of gonadotropin secretion in both sexes (25), and the AVPV is specifically implicated in mediating the positive feedback effects of estrogen on GnRH during the preovulatory LH surge (26, 27). Terminals projecting from cell bodies in the AVPV are found in close apposition to GnRH-containing perikarya (28); however, whether these terminals are derived from kisspeptin-54-expressing neurons whose cell bodies reside in the AVPV (or ARC) remains to be elucidated. Moreover, it will be of considerable interest to determine whether sex steroids regulate the KiSS-1 gene.

In summary, we have shown that KiSS-1 mRNA is expressed in regions of the hypothalamus known to be involved in the regulation of gonadotropin secretion, and products of the KiSS-1 gene have a potent stimulatory effect on GnRH secretion. We conclude that kisspeptins and their receptor are part of the hypothalamic circuitry that governs the neuroendocrine reproductive axis.

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