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Repetitive Activation of Hypothalamic G Protein-Coupled Receptor 54 with Intravenous Pulses of Kisspeptin in the Juvenile Monkey (*Macaca mulatta*) Elicits a Sustained Train of Gonadotropin-Releasing Hormone Discharges

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The purpose of the present study was to further examine the hypothesis that activation of G protein-coupled receptor 54 (GPR54) signaling at the end of the juvenile phase of primate development is responsible for initiation of gonadarche and the onset of puberty. Accordingly, we determined whether repetitive iv administration of the GPR54 receptor agonist kisspeptin-10 (2 μg as a brief 1-min infusion once every hour for 48 h) to the juvenile male rhesus monkey would prematurely elicit sustained, pulsatile release of hypothalamic GnRH, the neuroendocrine trigger for gonadarche. GnRH release was monitored indirectly by measuring LH secretion from the *in situ* pituitary, the GnRH responsiveness of which had been heightened before the experiment with an intermittent iv infusion of synthetic GnRH. Agonadal animals (n = 4) were employed to eliminate any confounding and secondary effects of changing feedback signals from the testis. The first brief infusion of kisspeptin-10 evoked an LH discharge that mimicked those produced by GnRH priming, and this was followed by a train of similar LH discharges in response to hourly activation of GPR54 by repetitive kisspeptin-10 administration. Concomitant treatment with a GnRH receptor antagonist, acyline, abolished kisspeptin-10-induced LH release. Repetitive kisspeptin-10 administration also provided a GnRH-dependent signal to FSH secretion. These findings are consistent with the notion that, in primates, the transition from the juvenile (attenuated GnRH release) to pubertal (robust GnRH release) state is controlled by activation of GPR54 resulting from increased expression of hypothalamic *KiSS-1* and release of kisspeptin in this region of the brain. (*Endocrinology* 147: 1007–1013, 2006)

PUBERTY IN MAN comprises two developmental processes, namely gonadarche and adrenarche (1). In primates, including man, gonadarche is triggered at the termination of the juvenile phase of development by a resurgence in the pulsatile secretion of GnRH from the hypothalamus (2). The neurobiology underlying this critical neuroendocrine event in primate development, however, remains an intriguing mystery. In this regard, the findings that hypothalamic GnRH mRNA and peptide levels in juvenile male and female monkeys are not dramatically different from those in animals in which the pubertal resurgence in GnRH release has occurred (3–6), and that a sustained adult mode of pulsatile GnRH release may be immediately provoked from the hypothalamus of the juvenile monkey by intermittent *N*-methyl-D-aspartic acid (NMDA) receptor activation (7), suggests that the signal for pubertal GnRH release must lie upstream from the GnRH neuronal network.

New, and perhaps fundamental, insight into the nature of the neurobiological signal responsible for the pubertal resurgence in GnRH release in primates has emerged as a result of the recently described association in man between inactivating mutations in G protein-coupled receptor 54 (GPR54) and hypogonadotropic hypogonadism with delayed puberty (8–10). GPR54 is expressed by GnRH neurons (11–13), and the ligands for this receptor are derived from the kisspeptins, which are encoded by *KiSS-1* (14–16). The view that GPR54 signaling may represent an important component of the trigger for pubertal GnRH release was substantiated by studies of the monkey demonstrating that expression of *KiSS-1* in pubertal females and agonadal pubertal males was greater than that in juvenile animals and that precocious GnRH release in juvenile male monkeys, equivalent in age to approximately 6-yr-old boys, was readily elicited by iv or intracerebroventricular bolus administration of a 10-amino-acid carboxyl-terminal fragment of kisspeptin (kisspeptin-10) (17).

If the transition from a quiescent GnRH neuronal network in the juvenile hypothalamus to a robustly pulsing network in the pubertal primate is the result of increased hypothalamic GPR54 signaling produced by an enhanced kisspeptin tone in this region of the brain, then premature and chronic activation of hypothalamic GPR54 in the juvenile by exogenous kisspeptin-10 administration should result prematurely in a sustained hypophysiotropic drive to the gonadotroph. To test this hypothesis, agonadal juvenile male rhesus monkeys, in which pituitary responsiveness to GnRH had been heightened with a priming infusion of synthetic GnRH before the study, received an uninterrupted intermittent iv

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Abbreviations: CCK, Cholecystokinin; DPBS, Dulbecco's PBS; GnRH-R, GnRH receptor; GPR54, G protein-coupled receptor 54; NMDA, *N*-methyl-D-aspartic acid.

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infusion of kisspeptin-10 for 48 h, and the effect on hypothalamic GnRH release was tracked indirectly by measuring circulating LH. The results of this study are presented here.

Materials and Methods

Animals

Four juvenile male rhesus monkeys (*Macaca mulatta*, 20–21 months of age, 2.6–3.8 kg body weight) were used. The ages of the animals at the end of the study ranged from 23–24 months; the pubertal reactivation of the hypothalamic-pituitary axis in this species occurs at around 30–36 months of age (2). The animals were maintained under controlled photoperiod (lights on from 0700–1900 h) and temperature (21 C) in accordance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee approved the experimental procedures.

Synthetic peptides

Synthetic, human kisspeptin-10 was either synthesized by the Peptide/Protein Core Facility of the Massachusetts General Hospital Endocrine/Reproductive Endocrine Unit and generously provided by Dr. William F. Crowley, Jr., or obtained commercially [*KiSS-1* (112–121-amide); Phoenix Pharmaceuticals, Inc., Belmont, CA). For the former, a stock solution of the peptide (350 μg/ml) was prepared in 5% dimethylsulfoxide in sterile physiological saline (0.9% NaCl) (Abbott Laboratories, North Chicago, IL), and for the latter, the stock (200 μg/ml) was prepared in sterile Dulbecco's PBS (DPBS without $CaCl_2$ and $MgSO_4$; Life Technologies, Inc. Products, Grand Island, NY); both were stored at −80 C. Working kisspeptin-10 infusate (2 μg/ml) was prepared by diluting a stock preparation in sterile DPBS, either the day before (stored at 4 C) or on the day of the experiment. Sterile DPBS was used for vehicle infusion.

The GnRH-receptor (GnRH-R) antagonist, acyline (300 μg/ml) (Bioqual, Rockville, MD), was prepared in 5% aqueous mannitol (AMVET Scientific Products, Yaphank, NY) and stored at 4 C (18). The antagonist was administered sc at a daily dose of 60 μg/kg, which has been shown to abolish GnRH-induced gonadotropin secretion (18). Sterile physiological saline was used as vehicle for the GnRH-R antagonist. GnRH (lot no. 230-110-40 BW) was synthesized at the Salk Institute (Contract N01-HD-0-2906) and obtained from the National Hormone and Peptide Program.

Surgical procedures

All surgeries were performed under sterile conditions. Bilateral castration and implantation of an iv catheter (inner diameter 0.040 in. and outer diameter 0.085 in) (Stuart Bio-Sil; Sil-Med Corp., Taunton, MA) were performed as described previously (18). Briefly, the animals were first sedated with ketamine hydrochloride (10–20 mg/kg body weight, im) (Ketaject; Phoenix Scientific Inc., St. Joseph, MO) and anesthetized by isoflurane inhalation (1–2%, in oxygen) (Abbott Animal House, North Chicago, IL). Bilateral castration was performed a few weeks before or at the time of catheterization. An indwelling catheter was placed either in an internal jugular or a femoral vein. The animals received a single im injection of penicillin (Pen-G, 40,000 U/kg body weight) (Phoenix Scientific) on the day of surgery. Postsurgically, the animals received twice-daily iv injections of a broad-spectrum antibiotic (Kefzol, 25 mg/kg body weight) (Apothecon, Princeton, NJ) and an analgesic (Ketofen, 2 mg/kg body weight) (Fort Dodge Animal Health, Fort Dodge, IA) for 4 d. The routine maintenance of animals in remote sampling cages has been described previously (19).

Administration of GnRH and kisspeptin-10

Brief infusions of GnRH or kisspeptin-10 were automatically introduced into the catheter every hour and immediately chased into the animal with a saline bolus (~1 ml/min for 3 min), as previously described (20). In this manner, a single iv catheter could be used for both infusion and sampling.

Collection of blood samples

Blood samples (0.6–1.0 ml) were withdrawn via the catheter into heparinized syringes and transferred to sterile tubes, and the plasma was harvested after centrifugation. During periods of sequential sampling, packed blood cells were resuspended with sterile saline and returned to the respective animal. Plasma was stored at −20 C until required for assay.

In situ GnRH bioassay

To use pituitary LH secretion as a bioassay for endogenous GnRH release in juvenile animals, the responsiveness of the gonadotrophs to GnRH stimulation was first enhanced by a chronic pulsatile iv infusion of GnRH (0.15 μg/min for 2 min in 1 ml every hour), as described on several occasions previously (19, 21, 22). A robust, adult-like LH response to exogenous GnRH stimulation is usually established by approximately 3–4 wk of pulsatile GnRH treatment (22). After termination of the priming infusion, circulating LH concentrations fall rapidly to undetectable levels, but the response of the pituitary to GnRH is maintained for several days (19), allowing experimentally induced endogenous GnRH release to be easily detected. GnRH priming was reinitiated between experiments.

Experimental design

Experiment 1: effect of an iv intermittent infusion of kisspeptin-10 on the secretion of GnRH/gonadotropins. Experiment 1 was initiated after confirmation that pituitary responsiveness to GnRH had been markedly up-regulated by intermittent priming with synthetic GnRH. At this time (d 1), the iv intermittent infusion of GnRH (1 pulse every hour) was interrupted and immediately replaced with an intermittent iv infusion of kisspeptin-10 (2 μg in 1 ml as a pulse of 1 min duration once every h for 48 h) or vehicle (DPBS; 1 ml for 1 min once every hour for 48 h) with two of the four monkeys receiving the kisspeptin-10 infusion. The dose of kisspeptin-10 was selected as a result of a pilot study in GnRH-primed agonadal males, in which a bolus injection of 2.5 μg kisspeptin-10 produced a discharge of LH that mimicked those generated by the GnRH priming infusion. Circulating concentrations of gonadotropins were monitored in frequent blood samples collected during interpulse intervals for 1) the last two pulses of the intermittent GnRH priming infusion on d 1, 2) the first three pulses of the intermittent kisspeptin-10 infusion on d 1, 3) three pulses of kisspeptin-10 on d 2, 4) the last two pulses of kisspeptin-10 on d 3, and 5) the first two GnRH pulses during initiation of GnRH repriming after termination of kisspeptin-10 treatment on the last day of the experiment. After a 2-wk interval, the experimental protocol was repeated adopting a crossover design.

Experiment 2: effect of an iv intermittent infusion of kisspeptin-10 on the secretion of GnRH/gonadotropins in the presence of a GnRH-R antagonist. This experiment was designed to confirm the conclusion of an earlier study (17) that kisspeptin-10-induced precocious gonadotropin secretion in the prepubertal monkey was stimulated indirectly by an action at the level of the hypothalamus. Approximately 2 wk after completion of experiment 1, during which time the priming infusion of GnRH was administered, experiment 2 was initiated (d 1). As in experiment 1, GnRH priming was again interrupted and immediately replaced with an intermittent infusion of kisspeptin-10 (2 μg in 1 ml once every hour for 48 pulses; n = 4). In contrast to experiment 1, animals received either a sc injection of GnRH-R antagonist or an injection of sterile saline on d 1, approximately 30 min after the first pulse of kisspeptin-10, and again at approximately 0900 h on the morning of d 2 and 3. Circulating concentrations of gonadotropins were monitored in blood samples collected according to a protocol identical to that employed for experiment 1. After completion of the kisspeptin-10 infusions, GnRH priming was reinitiated in all four animals, and after restoration of pituitary responsiveness was confirmed in the two GnRH-R antagonist-treated animals, the experimental protocol was repeated adopting a crossover design.

Assays

Plasma LH and FSH levels were measured using homologous (macaque) RIAs as described previously (23, 24). The sensitivity of the LH and FSH assays ranged between 0.21–0.34 and 1.26–2.65 ng/ml, re-

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spectively, and the mean intra- and interassay coefficients of variation for LH at approximately 70% binding were less than 6.5% and less than 14%, and for FSH at approximately 84% binding were less than 13% and less than 22.5%, respectively.

Statistical analysis

Average LH and FSH concentrations during windows of pulsatile GnRH priming and kisspeptin-10 or vehicle treatment were first derived for each animal, and these values were used to calculate the overall mean (± SEM) for each window. The significance of differences within and between treatments was determined for the mean hormone concentrations by multifactor ANOVA with repeated measures followed by Student-Newman-Keuls multiple range test (GB STAT Statistical Program, version 6.5.6 Pro; Dynamic Microsystems Inc., Silver Spring, MD). Hormone concentrations below the sensitivity of the assays were assigned a value equivalent to the minimum detectable concentration. Statistical significance was accepted at $P \leq 0.05$. All values are expressed as mean ± SEM.

Results

Experiment 1: effect of an iv intermittent infusion of kisspeptin-10 on the secretion of GnRH/gonadotropin

As expected, changes in circulating LH concentrations in response to intermittent hourly iv infusions of exogenous GnRH on d 1 was episodic with amplitudes of approximately 1 ng/ml superimposed on a basal level of 3 ng/ml (Fig. 1). Also, as expected, the first brief infusion of kisspeptin-10 1 h after withdrawing the pulsatile GnRH stimulation elicited a robust LH discharge that was similar in magnitude to that evoked by the GnRH priming infusion (Fig. 1). Subsequent intermittent iv administration of kisspeptin-10 sustained the pulsatile LH response without decrement throughout the 48-h duration of the kisspeptin-10 infusion (Fig. 1). As anticipated, circulating LH concentrations during vehicle treatment gradually declined to reach levels at or around the detectable limit of the assay on d 2 (Fig. 1).

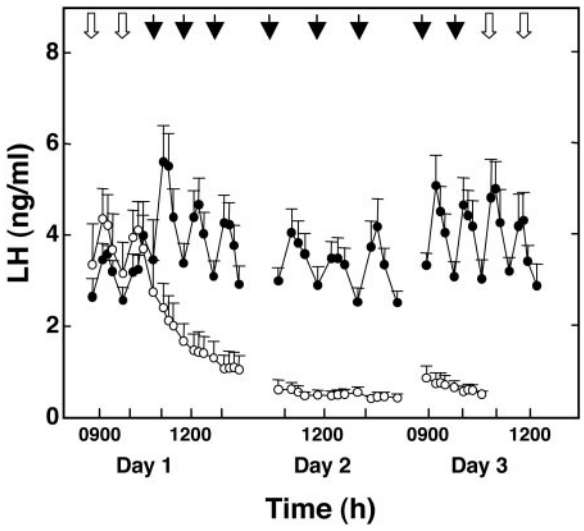

FIG. 1. Discharges of LH, as reflected by circulating concentrations of the gonadotropin, during the last two priming pulses of synthetic GnRH (*white arrows*) on d 1 in agonadal male rhesus monkeys were sustained without decrement by an intermittent iv infusion of 2 μg kisspeptin-10 per monkey administered as a 1-min pulse every hour for 48 h from 1100 h on d 1 to 1000 h on d 3 (*black data points*) after withdrawing GnRH treatment on d 1 but not during vehicle administration (*white data points*). Although kisspeptin-10 or vehicle was administered every hour, the LH response was tracked for only two or three pulses each day. The LH response to the reinitiation of the pulsatile GnRH priming infusion at 1100 h on d 3 was monitored after both kisspeptin-10 and vehicle treatment but shown only for the former treatment because of the exaggerated response in the vehicle group (see Fig. 3). This is presumably because of continued synthesis of LH in the absence of GnRH stimulation leading to a large releasable pool of the gonadotropin. *Black arrows* indicate times of pulse infusions of kisspeptin-10 or vehicle that were selected for tracking LH responses. *White arrows* indicate time of GnRH pulse infusions. Values are mean ± SEM.

The changes in circulating FSH concentrations during this experiment were qualitatively comparable to those of LH (Fig. 2), although pulsatile secretion of FSH was less conspicuous than that of LH, and the decline in FSH levels after vehicle administration was slower and less marked than that for LH (Fig. 1). During vehicle administration, FSH levels were maintained at approximately 50% of those during GnRH priming or subsequent kisspeptin-10 administration.

Figure 3 illustrates mean circulating concentrations of LH and FSH during 1) GnRH priming, 2) each day of kisspeptin-10 or vehicle infusions, and 3) GnRH repriming after the termination of kisspeptin-10 or vehicle treatment. The LH response to the first infusion of the GnRH repriming regimen at the end of vehicle treatment was markedly pronounced compared with that seen at the end of kisspeptin-10 infusion (Fig. 3).

Experiment 2: effect of an iv intermittent infusion of kisspeptin-10 on the secretion of GnRH/gonadotropin in the presence of GnRH-R antagonist

The time course of the LH responses to the last two intermittent priming infusions of GnRH and the first pulse of

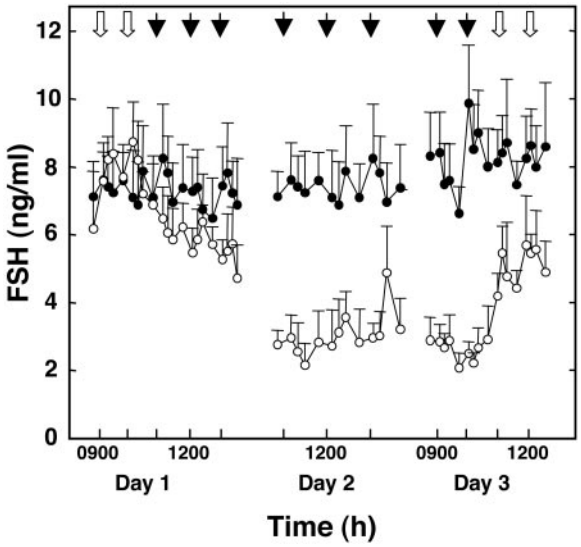

FIG. 2. Circulating FSH concentrations (mean ± SEM) during the last two priming pulses of synthetic GnRH (*white arrows*) on d 1 in agonadal male rhesus monkeys were sustained without decrement by an intermittent iv infusion of 2 μg kisspeptin-10 per monkey administered as a 1-min pulse every hour for 48 h from 1100 h on d 1 to 1000 h on d 3 (*black data points*) after withdrawing GnRH treatment on d 1 but not during vehicle administration (*white data points*). Although kisspeptin-10 or vehicle was administered every hour, the LH response was tracked for only two or three pulses each day. The LH response to the reinitiation of the pulsatile GnRH priming infusion at 1100 h on d 3 was monitored after both kisspeptin-10 and vehicle treatment. *Black arrows* indicate times of pulse infusions of kisspeptin-10 or vehicle that were selected for tracking LH responses.

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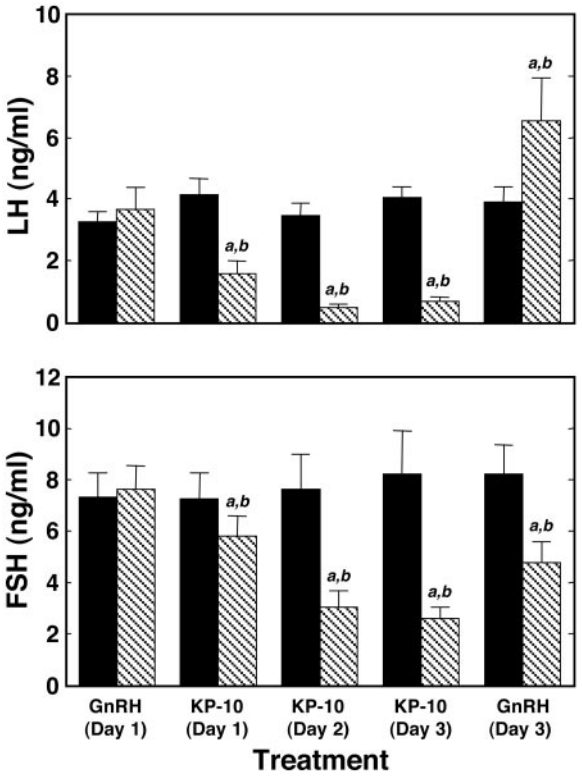

FIG. 3. Mean (± SEM) circulating concentrations of LH (*top*) and FSH (*bottom*) in agonadal juvenile male monkeys during the last two priming GnRH pulses on d 1, during intermittent iv infusion of kisspeptin-10 (KP-10; 2 μg/monkey every hour for 48 h) on d 1–3, and during the first two repriming pulses of GnRH on d 3 (*black bars*). Corresponding values for vehicle treatment are shown by *cross-hatched bars*. a, Significantly different from GnRH d 1; b, significantly different from treatment.

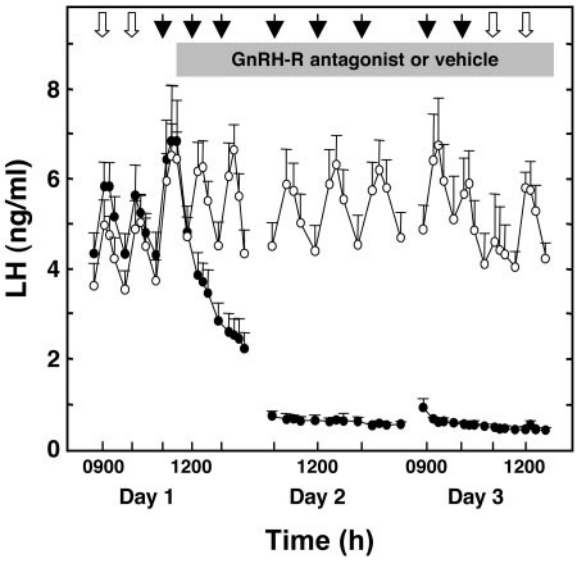

FIG. 4. Pulsatile LH release induced in agonadal juvenile male monkeys by an intermittent iv infusion of kisspeptin-10 (2 μg/animal as a brief 1-min infusion every hour for 48 h) starting at 1100 h on d 1 was abolished by treatment with a GnRH-R antagonist initiated immediately after the first pulse of the intermittent kisspeptin-10 infusion (*black data points*). Although kisspeptin-10 was administered every hour, the LH response was tracked for only two or three pulses each day. Treatment of the animals with a sc injection of saline on d 1 (vehicle used for GnRH-R antagonist treatment) did not influence kisspeptin-10-induced LH discharges (*white data points*). The LH response to reinitiation of the pulsatile GnRH priming infusion at 1100 h on d 3 was monitored for both GnRH-R antagonist and vehicle-treated kisspeptin-10-stimulated monkeys. Values are mean ± SEM. *Black arrows* indicate times of pulse infusions of kisspeptin-10 that were selected for tracking LH responses; *white arrows* indicate times of GnRH pulse infusions.

kisspeptin-10 was episodic and similar to that observed in experiment 1 (Fig. 4). Administration of the GnRH-R antagonist approximately 30 min after the first pulse of kisspeptin-10, however, resulted in the loss of episodic LH release and in a gradual, but significant, decline in the concentration of this gonadotropin that, by d 2, reached levels at or around the detectable limit of the assay where they were maintained for the duration of intermittent kisspeptin-10 treatment (Fig. 4). Moreover, the LH response to the resumption of pulsatile GnRH priming at the end of the kisspeptin-10 infusion was abolished (Fig. 4). Injection of saline (vehicle used for GnRH-R antagonist) on d 1 was without effect on the LH response during the 48-h kisspeptin-10 infusion (Fig. 4).

The impact of GnRH-R antagonism on kisspeptin-10-induced FSH release was less dramatic than that on LH secretion (Figs. 5 and 6). Significant differences between antagonist and vehicle treatment in FSH concentrations did not emerge until d 2, and although mean FSH levels on d 2 and 3 during treatment with the antagonist were significantly lower than that during vehicle administration, they were nevertheless maintained at values of approximately 50% of those in the control condition (Figs. 5 and 6).

Discussion

The present finding that an intermittent iv infusion of kisspeptin-10 elicited a train of GnRH-dependent LH discharges in the agonadal juvenile male monkey, in which pituitary responsiveness to GnRH had been heightened by previous exposure to a pulsatile infusion of synthetic GnRH, supports our earlier conclusion that premature activation of GPR54 within the hypothalamus of the prepubertal primate leads to precocious release of GnRH (17) and extends the latter study, which examined only the action of isolated stimulation with kisspeptin-10. First, repetitive kisspeptin-10 stimulation of the hypothalamic-pituitary unit of the prepubertal monkey at hourly intervals for 48 h induced a sustained train of LH discharges that mimicked those produced by the pulsatile infusion of synthetic GnRH administered immediately before stimulation with kisspeptin-10. Because the frequency and magnitude of the LH discharges produced in agonadal juvenile males by GnRH priming are, to a first approximation, similar to those observed spontaneously in castrated adult male monkeys (25), it is reasonable to conclude that the GnRH priming infusion provides the gonadotrophs of the juvenile pituitary with a hypophysiotropic stimulus comparable to that produced spontaneously by the adult hypothalamus unrestrained by negative feedback signals from the testis. By extension, it may be further proposed that repetitive activation of GPR54 in the hypothalamus of the prepubertal male monkey elicited a precocious and sustained hypophysiotropic drive to the pituitary gonadotroph that mimicked that observed spontaneously in the castrated

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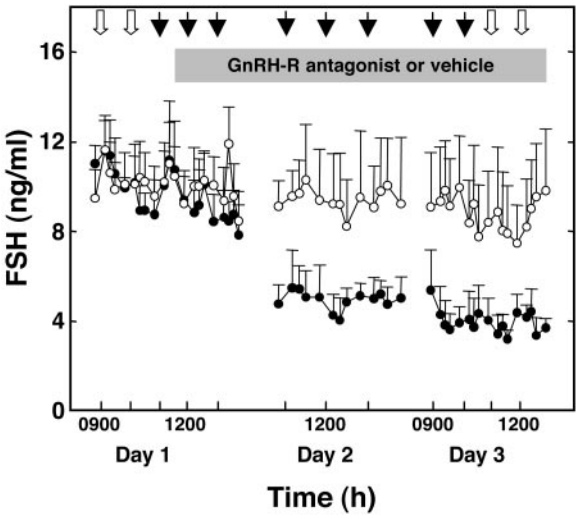

FIG. 5. Circulating FSH concentrations (mean ± SEM) in agonadal juvenile male monkeys induced by an intermittent iv infusion of kisspeptin-10 (2 μg/animal as a brief 1-min infusion every hour for 48 h) starting at 1100 h on d 1 were reduced by treatment with a GnRH-R antagonist initiated immediately after the first pulse of the intermittent kisspeptin-10 infusion (*black data points*). Although kisspeptin-10 was administered every hour, the FSH response was tracked for only two or three pulses each day. Treatment of the animals with a sc saline injection on d 1 (vehicle used for GnRH-R antagonist treatment) did not influence kisspeptin-10-induced FSH levels (*white data points*). The FSH response to reinitiation of the pulsatile GnRH priming infusion at 1100 h on d 3 was monitored for both GnRH-R antagonist and vehicle-treated kisspeptin-10-stimulated monkeys. *Black arrows* indicate times of pulse infusions of kisspeptin-10 that were selected for tracking LH responses; *white arrows* indicate times of GnRH pulse infusions.

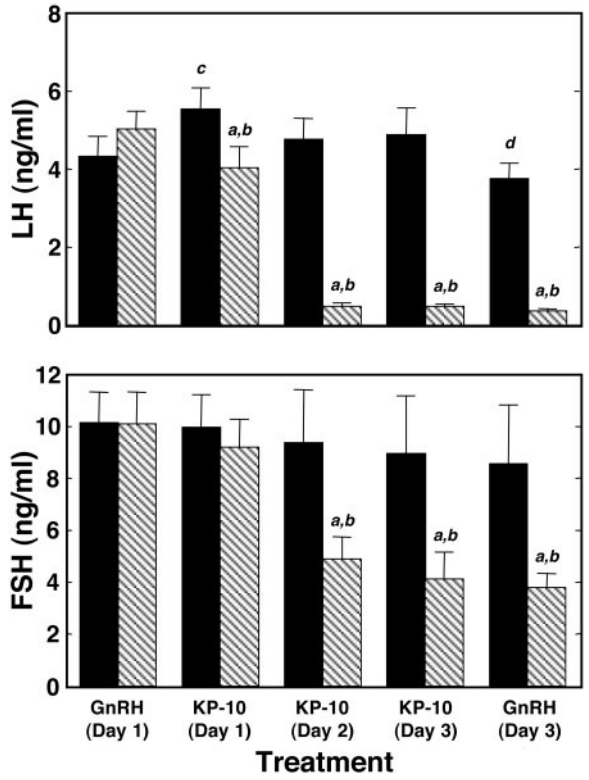

FIG. 6. Mean (± SEM) circulating concentrations of LH (*top*) and FSH (*bottom*) in agonadal juvenile male monkeys during intermittent iv infusion of kisspeptin-10 (KP-10) in the presence (*cross-hatched bars*) or absence (*black bars*) of GnRH-R antagonist treatment. For comparison, mean circulating concentrations of gonadotropins produced in response to GnRH priming immediately before and after the kisspeptin-10 infusion in the presence or absence of GnRH-R antagonist treatment are also shown. a, Significantly different from GnRH on d 1; b, GnRH antagonist treatment significantly different from vehicle; c, significantly different from GnRH on d 1; d, significantly different from kisspeptin-10 on d 1–3.

adult. In the undernourished female rat, central administration of kisspeptin every 12 h from d 30–37 of age reversed the delay in vaginal opening resulting from the nutritional insult, indicating that GnRH neurons in the rodent are also capable of responding to repetitive GPR54 activation (26). Whether the hypothalamic site of action of iv-administered kisspeptin 10 in the monkey is inside or outside the blood-brain barrier remains to be established.

Precocious GnRH release in the juvenile male rhesus monkey has also been induced by iv administration of cholecystokinin (CCK), another centrally active peptide (27). Although the effect of prolonged repetitive stimulation with CCK has not been studied, when a total of three sequential injections of CCK were administered at intervals of 2 h, the magnitude of the response to the second and third injection was less than that to the first challenge (27). We therefore consider it unlikely that a sustained pulsatile release of GnRH would be produced over a 2-d period by hourly stimulation with CCK, as it was in the present study by repetitive kisspeptin-10 administration.

The ability of repetitive hourly activation of hypothalamic GPR54 in the juvenile monkey to elicit robust trains of GnRH discharges without evidence of decrement is reminiscent of the action of NMDA (7), which, when administered in a pulsatile manner for several months, drives the hypothalamic-pituitary-testicular axis of the juvenile monkey into an adult mode of operation with onset of episodic testicular testosterone secretion and the initiation of spermatogenesis (28). We would predict that similar chronic repetitive stimulation of hypothalamic GPR54 in the juvenile monkey would also result in precocious gonadarche, but this remains to be demonstrated empirically.

The foregoing discussion raises the question as to what, if any, is the likely relationship between GPR54 and NMDA receptor signaling at the time of initiation of the spontaneous pubertal resurgence of pulsatile GnRH release in primates, the key neuroendocrine event triggering the onset of gonadarche in these species (2). In this regard, it is to be noted that an increase in hypothalamic glutamate levels has been reported at the time of gonadarche in the monkey, albeit to date only in the female (29), and an increase in kisspeptin release is to be anticipated at this stage of development because *KiSS-1* expression is up-regulated at this time (17). In a recent study of signaling pathways regulating secretion by the rat GnRH neuron, Tena-Sempere and his colleagues (30) reported that the GnRH-releasing action of kisspeptin was not blocked by previous treatment with MK 801, a noncompetitive NMDA antagonist. If the latter findings apply also to the monkey, it may be suggested either that the NMDA receptor and GPR54 are components of independent signaling pathways afferent to the GnRH neuron or that glutamate acti-

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vation of GnRH is mediated indirectly via *KiSS-1*-expressing neurons proximal to the GnRH neuronal network. The former possibility is consistent with the finding that GPR54 is colocalized with GnRH in the hypothalamus of both the rodent and primate (12, 13) and that glutamate receptors, including the NMDA subtype, are expressed by GnRH neurons, at least in the rat (31–35). Glutamate neurotransmission in the brain is ubiquitous, and the action of NMDA on the neuroendocrine hypothalamus does not appear to be restricted to the neuronal system regulating GnRH release because central or peripheral administration of this agonist results in GH, prolactin, and ACTH release (21, 28, 36). On the other hand, *KiSS-1* expression in the hypothalamus is restricted primarily to the region of the arcuate nucleus (17), and administration of kisspeptin-10 to the monkey has not been observed to provoke consistent changes in either GH or prolactin secretion (Shahab, M., and T. M. Plant, unpublished observations). Taking the foregoing considerations together, we would propose that the role of hypothalamic kisspeptinergic neurons in the developmental regulation of GnRH release is fundamental, whereas that of glutamatergic interneurons may be subsidiary. The availability of GPR54 antagonists will be required to pursue this and other hypotheses further.

Initial studies of the neuroendocrine action of kisspeptin-10 in the monkey employed bolus iv or intracerebroventricular injections of 100 μg kisspeptin-10 (17). Regardless of the route of administration, similar discharges of LH were evoked, suggesting that the response to this dose of the GPR54 agonist was supramaximal. For the present study, the kisspeptin-10 dose was reduced by 50-fold, and as discussed above, this dose appeared to elicit a physiological discharge of GnRH from the monkey hypothalamus. As also discussed above, CCK at doses of 10–30 μg/kg (*i.e.* 9–26 nmol/kg) was able to elicit GnRH release in the juvenile monkey. This dose range for CCK may be compared with the kisspeptin-10 dose of approximately 0.67 μg/kg (0.5 nmol/kg) employed in the present study. Therefore, as described for other species (37–39), the sensitivity of GnRH release to GPR54 activation in response to kisspeptin-10 is remarkable.

In man and monkey, it is generally recognized that a hypophysiotropic drive comprising GnRH, alone, is sufficient for the onset of puberty and for the maintenance of adult gonadal function in both the male and female, which requires the concomitant secretion of LH and FSH. It is not surprising, therefore, that in the present study repetitive administration of kisspeptin-10 maintained circulating FSH at levels noted during the GnRH priming infusion. That kisspeptin-10-induced FSH release, like that of LH, was GnRH dependent was indicated by the finding that when the GnRH-R was blocked during kisspeptin-10 administration, FSH levels were indistinguishable from those observed during the 48-h period after termination of GnRH priming. A similar GnRH-dependent action of kisspeptin-10 on FSH release has recently been described for the rat (40). The maintenance of FSH levels of 2–4 ng/ml after withdrawal of GnRH priming in the present study most likely reflects differences in 1) the kinetics at which the synthesis and release of FSH and LH is interrupted after withdrawal of GnRH stimulation and 2) clearance of the gonadotropins from the circulation. Before the initiation of GnRH priming, circulating FSH is usually undetectable ($\leq$0.1 ng/ml) in juvenile male rhesus monkeys (41).

GPR54 is expressed in the pituitary in rodents, monkey, and man (Refs. 14, 16, 42; and Shibata, M., and T. M. Plant, unpublished observations), and modest stimulation of LH release from cultures of rat pituitary fragments has been reported in response to addition of kisspeptin (30). A direct effect of kisspeptin-10 at the pituitary level in the present study, however, is unlikely because GnRH-R antagonist treatment abolished kisspeptin-10-induced LH release.

In conclusion, the findings reported here are consistent with the hypothesis that, in higher primates, a reduced kisspeptin tone in the hypothalamus represents a major component of the prepubertal brake on pulsatile GnRH releas, and that the pubertal resurgence in GnRH release that induces gonadarche is triggered by enhanced GPR54 signaling to GnRH neurons resulting from up-regulation of *KiSS-1* expression and increased kisspeptin release. Although the relationship between the pubertal increase in GPR54 signaling and the concomitant decreases in hypothalamic neuropeptide Y and γ-aminobutyric acid tone that have been, respectively, established for the male and female monkey (5, 43) remains to be determined, it is tempting to speculate that the enhanced kisspeptin tone at the termination of the juvenile phase of development may result from a waning of these inhibitory inputs. However the case may be, the physiological control system that times the neurochemical events that lead to the pubertal resurgence of GnRH release in primates, and therefore the onset of puberty in these species, remains a fascinating mystery.

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T.M.P., S.R., and M.J.D. have nothing to declare.

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