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Continuous Human Metastin 45–54 Infusion Desensitizes G Protein-Coupled Receptor 54-Induced Gonadotropin-Releasing Hormone Release Monitored Indirectly in the Juvenile Male Rhesus Monkey (*Macaca mulatta*): A Finding with Therapeutic Implications

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The effect of continuous administration of the C-terminal fragment of metastin, the ligand for the G protein-coupled receptor, GPR54, on GnRH-induced LH secretion was examined in three agonadal, juvenile male monkeys whose responsiveness to GnRH was heightened by pretreatment with a chronic pulsatile iv infusion of synthetic GnRH. After bolus injection of 10 μg human (hu) metastin 45–54 (equivalent to kisspeptin 112–121), the GPR54 agonist was infused continuously at a dose of 100 μg/h and elicited a brisk LH response for approximately 3 h. This rise was then followed by a precipitous drop in LH despite continuous exposure of GPR54 to metastin 45–54. On d 4, during the final 3 h of the infusion, single boluses of hu metastin 45–54 (10 μg), *N*-methyl-DL-aspartic acid (NMDA) (10 mg/kg) and GnRH (0.3 μg) were administered to interrogate each element of the metastin-GPR54-GnRH-GnRH receptor cascade. Although the NMDA and GnRH boluses were able to elicit LH pulses, that of hu metastin 45–54 was not, demonstrating functional integrity of GnRH neurons (NMDA) and GnRH receptors (NMDA and GnRH) but desensitization of GPR54. The desensitization of GPR54 by continuous hu metastin 45–54 administration has therapeutic implications for a variety of conditions currently being treated by GnRH and its analogs, including restoration of fertility in patients with abnormal GnRH secretion (*i.e.* idiopathic hypogonadotropic hypogonadism and hypothalamic amenorrhea) and selective, reversible suppression of the pituitary-gonadal axis to achieve suppression of gonadal steroids (*i.e.* precocious puberty, endometriosis, uterine fibroids, and prostate cancer). (*Endocrinology* 147: 2122–2126, 2006)

THE TRIGGERS FOR the resurgence of GnRH secretion at the time of puberty in primates are as mysterious as those that halt its secretion at the end of the infantile period. Several approaches have been employed to identify these elusive signals, including genetic studies using DNA from patients with reproductive disorders. Loss-of-function mutations in the gene encoding the G protein-coupled receptor, GPR54, have recently been demonstrated to cause hypogonadotropic hypogonadism, a condition characterized by an absence of pubertal development (1, 2). Across species, *Gpr54* knockout mice are phenocopies of this syndrome (2).

The endogenous ligand of GPR54 is derived from kisspeptin-1, which is proteolytically processed in man to a 54-amino-acid peptide, human (hu) metastin (3–5). Metastin's name was so coined because of its ability to suppress metastases of human melanomas and breast carcinomas (6, 7). When administered as a single bolus to mice (8), rats (9–13), and agonadal, juvenile, male monkeys (14), hu metastin and hu metastin 45–54 (or the mouse analog) elicit a robust LH response that is blocked by previous treatment with a GnRH receptor antagonist and, therefore, presumably mediated through GnRH release from the hypothalamus (8, 14). When administered as an iv infusion to ovariectomized, estradiol-treated sheep (4 h), hu metastin 45–54 also stimulates LH release and GnRH levels are increased in cerebrospinal fluid (15). In human males, an iv infusion (90 min) of hu metastin also stimulates LH release (16). Outside of these single boluses and brief infusions, chronic, intermittent administration of hu metastin 45–54 (or the mouse analog) induces early sexual maturation in immature female rats (13) and sustained and precocious GnRH release in juvenile male monkeys (17). However, the effect of long-term continuous metastin administration on the hypophysiotropic drive to the gonadotrophs is unknown. The goal of this study was to test the hypothesis that administration of a 4-d, continuous infusion of high-dose hu metastin 45–54 to juvenile, agonadal male rhesus monkeys stimulates sustained gonadotropin release and, by extension, sex steroid secretion.

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Abbreviations: DMSO, Dimethylsulfoxide; DPBS, Dulbecco's PBS; GPR, G protein-coupled receptor; hu, human; NMDA, *N*-methyl-DL-aspartic acid.

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Materials and Methods

Animals

Three juvenile male rhesus monkeys (*Macaca mulatta*, 19–20 months of age, 2.6–3.8 kg body weight) were used. The age of the animals at the

end of the study was 21–23 months, and the pubertal reactivation of the hypothalamic-pituitary axis under the conditions of the present experiment usually occurs from 24–30 months of age (18). The animals were maintained under controlled photoperiod (lights on from 0700–1900 h) and at approximately 21 C in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The experimental procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Reagents

Metastin, the ligand for GPR54, is proteolytically processed from its parent protein, kisspeptin-1. This paper employs the term metastin with amino acid numbering reflecting this protein/peptide specifically (C-terminal fragment of hu metastin 45–54 is equivalent to hu kisspeptin-1 112–121). The C-terminal fragment of hu metastin (hu metastin 45–54) was selected for these studies because it has been shown to have comparable biological potency as the full-length peptide (4). The hu metastin 45–54 dose chosen for continuous administration (100 μg/h iv) was based on previous studies (14) in which bolus iv injections of 100 μg of this peptide were found to stimulate GnRH-induced LH secretion in agonadal, juvenile monkeys. Hu metastin 45–54 was synthesized at the Peptide/Protein Core Facility of the Massachusetts General Hospital. A stock solution of the peptide (500 μg/ml) was prepared in 5% dimethylsulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO) in physiological saline (0.9% NaCl) (Abbott Laboratories, Chicago, IL) and stored at −80 C. For continuous administration, the hu metastin 45–54 infusate (100 μg/3 ml) was prepared the day before the experiment began by diluting the stock preparation with sterile Dulbecco's PBS (DPBS) without $CaCl_2$ and $MgSO_4$ (Life Technologies, Inc. Products, Grand Island, NY) and stored at 4 C. During the experiment, a calibrated reservoir (Buretrol; Baxter Healthcare Corp., Deerfield, IL) containing the infusate was maintained at room temperature and refilled every 24 h. A stock 5% DMSO solution in sterile saline was prepared and stored at −80 C. For vehicle infusion, this was diluted 1:15 with sterile DPBS, stored at 4 C, and used as described for the hu metastin 45–54 infusate.

For bolus administration of hu metastin 45–54, a 10-μg dose (10 μg/ml in sterile DPBS) was used. This bolus dose was chosen, in part, because of the recent demonstration that repetitive 2-μg boluses of hu metastin 45–54 administered once every hour are capable of inducing LH discharges in agonadal, juvenile male monkeys (17). However, because a 2-μg bolus of hu metastin 45–54 infused over 1 min is roughly equivalent to the continuous infusion rate (100 μg/h = 1.7 μg/min), a higher dose of 10 μg was chosen for the bolus challenges in this experiment. *N*-Methyl-DL-aspartic acid (NMDA) (Sigma-Aldrich Inc., St. Louis, MO) was dissolved in sterile saline at a stock concentration of 50 mg/ml. On the days of NMDA administration, doses of 10 mg/kg body weight were prepared in 1 ml sterile saline and passed through a 0.22-μm filter (Fisher Scientific, Pittsburgh, PA) before injection.

GnRH, synthesized at the Salk Institute (Contract N01-HD-0-2906), was obtained from the National Hormone and Peptide Program. A stock GnRH solution was prepared at 1 mg/ml in sterile saline and stored at −20 C. For intermittent infusion, GnRH was diluted to 0.3 μg/ml in saline, stored at −20 C, and used as required.

Surgical procedures

Bilateral castration and implantation of iv catheters (inner diameter, 0.040 in; outer diameter, 0.085 in) (Stuart Bio-Sil; Sil-Med Corp., Taunton, MA) were performed under sterile conditions as described previously (18). Briefly, the animals were 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. Two indwelling catheters were employed, one placed in an internal jugular or subclavian vein and the other in a femoral vein. During the continuous infusion of hu metastin 45–54, one line was dedicated to infusion and one to sampling. 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 (18).

Collection of blood samples

Blood samples (1 ml) were withdrawn via the sampling 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 assays.

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 every hour), as described on several occasions previously (14, 18–21). A robust, adult-like LH response to exogenous GnRH stimulation is usually established by approximately 3–4 wk of pulsatile GnRH treatment (21). 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 (18), allowing experimentally induced endogenous GnRH release to be easily detected. GnRH priming was reestablished between the hu metastin 45–54 and vehicle infusions.

Experimental design

The experiment was initiated after 4–5 wk of intermittent priming with GnRH and after confirmation that pituitary responsiveness to GnRH had been markedly up-regulated by this treatment. At this time (d 1), the iv intermittent infusion of GnRH was interrupted. One hour after the last priming pulse of GnRH, 10 μg hu metastin 45–54 was administered as a bolus iv injection, and 1 h later the continuous iv infusion of hu metastin 45–54 (100 μg/h for 98 h) was initiated. The volume of infusion was monitored on a daily basis. During the last 3 h of the continuous hu metastin 45–54 infusion on d 4, the animals received, in sequence, a bolus injection of 10 μg hu metastin 45–54, a bolus injection of NMDA (10 mg/kg body weight), and a bolus injection of GnRH. In one monkey, the bolus injection of hu metastin 45–54 was administered after the GnRH and NMDA challenge. One day after termination of the continuous hu metastin 45–54 infusion, the animals received another iv bolus of 10 μg hu metastin 45–54. Finally, the intermittent priming infusion of GnRH was reinitiated.

Circulating concentrations of LH were monitored in blood samples collected on the following occasions. 1) On d 1, samples were collected before and after the last GnRH priming pulse and the bolus of hu metastin 45–54 (during these times, series of blood samples were collected 10 min before and at 10, 20, 30, and 50 min after the peptide bolus). Samples were also collected on d 1 during the first 12 h of the continuous hu metastin 45–54 infusion (at 10, 20, 30, 50, 70, 90, 110, 130, 150, 170, 360, and 720 min into the infusion). 2) On d 2, 3, and 4 of the continuous hu metastin 45–54 infusion, a single blood sample was collected in the morning and evening, at approximately 1000 and 2200 h, respectively. In addition, in two of three animals, a nocturnal series of blood samples was collected on d 2 at 20-min intervals over a 3-h period (1900–2200 h). 3) On d 4 of the continuous hu metastin 45–54 infusion, series of blood samples were collected to describe the LH response to bolus injections of hu metastin 45–54, NMDA, and GnRH. 4) One day after termination of the continuous hu metastin 45–54 infusion, a series of blood samples were collected before and after another iv bolus of hu metastin 45–54.

Nonheparinized blood samples were also collected in EDTA tubes before, during, and after the continuous hu metastin 45–54 infusion to measure peptide levels in the circulation at a later date. Plasma samples were stored at −20 C or below.

After a 1- to 3-wk interval, during which time the animals were reprimed with pulsatile GnRH, the control experiment was performed using a continuous infusion of vehicle (0.33% DMSO in sterile DPBS at 3 ml/h for 98 h) employing an essentially identical protocol.

LH assays

Plasma LH levels were measured using a homologous (macaque) RIA as described previously (21, 22). The sensitivity of the LH assay ranged from 0.36–0.42 ng/ml, and the intra- and interassay coefficients of variation for LH at 74% binding were less than or equal to 3.5 and 13.6%, respectively.

Statistical analysis

The significance of differences between mean LH concentrations were examined with the Student's *t* test, using Sigma Stat.

Results

Effect of continuous administration of hu metastin 45–54 on LH release in agonadal juvenile male monkeys

The last iv priming pulse of GnRH administered to three agonadal male monkeys at 0800 h on d 1 induced an LH discharge that increased circulating LH levels from 4.2 ± 0.7 ng/ml to a peak level of 5.8 ± 0.1 ng/ml (mean ± SEM; see Fig. 1). At 0900 h, the iv administration of 10 μg hu metastin 45–54 also elicited a rise in plasma LH levels, the amplitude (basal to peak, 4.7 ± 1.6 to 8.8 ± 1.7 ng/ml) of which was almost 2-fold that produced by the preceding bolus of GnRH. At 1000 h, continuous exposure to hu metastin 45–54 (100 μg/h) was initiated. Peak LH levels (10.6 ± 0.8 ng/ml) were observed at 1–2 h, and these then declined dramatically in the face of continuing exposure to the peptide, reaching, within 12 h, values (~1 ng/ml) indistinguishable from the control vehicle infusions. Interestingly, these very low LH levels were sustained during the day (1000 h), but in the evening (2200 h), modest, albeit nonsignificant, elevations were consistently observed during both continuous hu metastin 45–54 and vehicle infusions (Fig. 2).

Effect of single boluses of hu metastin 45–54, NMDA, and GnRH on LH release during the last 3 h of the continuous 4-d administration of hu metastin 45–54 in agonadal juvenile male monkeys

On d 4, single doses of hu metastin 45–54, NMDA, and GnRH were administered iv during the final 3 h of the hu metastin 45–54 infusion (Fig. 3). Although NMDA and GnRH elicited discharges of LH, hu metastin 45–54 did not. Twenty-one hours after termination of the metastin 45–54 infusion, however, a profound LH response was induced by administration of an identical bolus of the peptide.

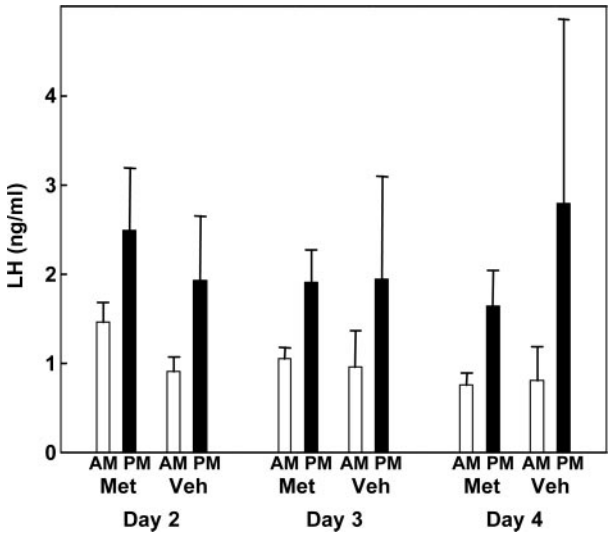

FIG. 2. Daytime (0900 h, *white bars*) and nighttime (2200 h, *black bars*) plasma LH levels (mean ± SEM) from three monkeys on d 2, 3, and 4 of a continuous infusion of 100 μg/h hu metastin 45–54 (Met) or vehicle (Veh). Daytime *vs.* nighttime differences were not significant by paired *t* test ($P > 0.05$).

Discussion

Reminiscent of intermittent GnRH infusions to hypothalamically lesioned monkeys (23), we recently demonstrated that intermittent pulsatile hu metastin 45–54 administration (2 μg per pulse at a circhoral frequency) drives gonadotropin secretion in agonadal, juvenile male monkeys previously primed with GnRH (17). In striking contrast, as shown in the present study with a similar experimental model, continuous

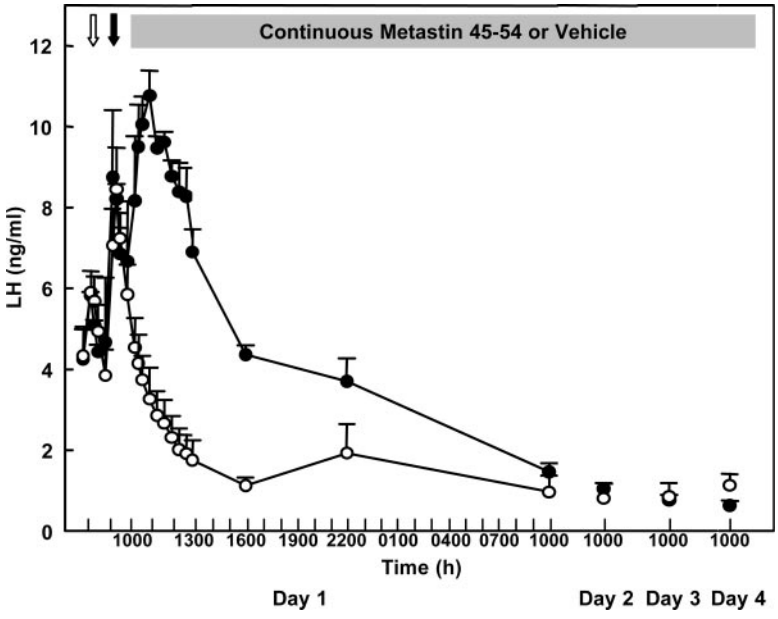

FIG. 1. Effect of continuous administration of hu metastin 45–54 on LH release in agonadal juvenile male rhesus monkeys, in which pituitary responsiveness to GnRH had been previously heightened by pulsatile GnRH treatment. ●, LH levels (mean ± SEM) from three monkeys receiving 100 μg/h hu metastin 45–54 over a 98-h infusion period (*shaded horizontal bar*); ○, mean LH levels during vehicle infusion. Administration of hu metastin 45–54 or vehicle was initiated at 1000 h on d 1. The *white arrow* indicates iv administration of the last GnRH priming pulse at 0800 h on d 1. The *black arrow* indicates iv administration of single bolus of hu metastin 45–54 at 0900 h on d 1.

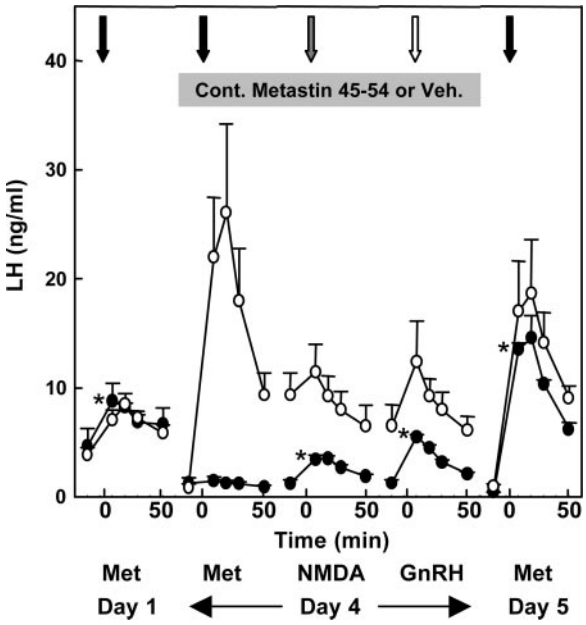

FIG. 3. Effect of single sequential boluses of hu metastin 45–54 (Met) (*black arrow*), NMDA (*gray arrow*), and GnRH (*white arrow*) on plasma LH concentrations (mean ± SEM) during the last 3 h of the 98-h iv infusion (*shaded horizontal box*) of hu metastin 45–54 at a dose of 100 μg/h (●) or vehicle (Veh) (○) compared with the LH response to the same bolus of hu metastin 45–54 1 h before (d 1) and 21 h after (d 5) the termination of continuous hu metastin 45–54 or vehicle infusion. *, Infusion of hu metastin 45–54 (●) significantly different ($P < 0.05$) from preinjection mean; n = 3.

administration of hu metastin 45–54 at 100 μg/h abolishes the LH response after an initial, acute stimulatory effect.

Possibilities to explain the observed decrease in LH levels in the face of continuous exposure to hu metastin 45–54 include desensitization of GPR54 to the exogenous metastin, depletion of GnRH from the GnRH neurons, desensitization of the GnRH receptor to GnRH, or depletion of LH from the pituitary gonadotrophs. Single boluses of several agonists were administered during the final day of the hu metastin 45–54 infusion to test these hypotheses. On d 4 of the hu metastin 45–54 infusion, administration of NMDA, an excitatory amino acid analog, evoked a robust LH response, demonstrating adequate stores of releasable GnRH within GnRH neurons, intact GnRH receptor signaling, and adequate stores of LH in the gonadotrophs. Administration of a 0.3-μg physiological pulse of GnRH also elicited a robust LH response (17), again testifying to the retained signaling capacity of the GnRH receptor and intact LH stores. However, administration of a single bolus of hu metastin 45–54 (concomitant with the continuous hu metastin 45–54 infusion), at a dose that elicited a robust LH discharge at the beginning of the experiment, failed to evoke a LH response. These data suggest that the inability to maintain LH levels 6 h after continuous hu metastin 45–54 infusion is due to desensitization or down-regulation of GPR54. Although the cellular mechanism for such an effect is currently unknown, sensitivity to exogenous hu metastin 45–54 was restored 21 h after its continuous administration was stopped.

Two other studies have used a metastin infusion paradigm. Fifty nanomoles of hu metastin 45–54 were infused intracerebroventricularly over 4 h to adult ovariectomized ewes treated with estradiol implants (15). Although GnRH levels in cerebrospinal fluid were sustained during the course of the infusion, LH levels appeared to wane by the end of the treatment period in some animals. If GnRH levels in cerebrospinal fluid accurately reflect those in portal blood, the latter finding suggests the development of gonadotroph refractoriness. This observation would then stand in contrast to our data indicating that pituitary responsivity to exogenous GnRH remained intact. In addition to species- and sex-specific mechanisms, differences between the two models include 1) the sex steroid milieu (the monkeys used in these experiments were juvenile and gonadectomized with no sex-steroid treatment) and 2) the dose and duration of metastin. In addition to the sheep study, recently, full-length hu metastin 1–54 was infused briefly (90 min) in healthy male volunteers (16). LH, FSH, and testosterone levels rose significantly, but additional interrogations of the hypothalamic-pituitary-gonadal axis were not performed.

Despite the dramatic drop in LH levels that occurred on d 1 of the hu metastin 45–54 infusion reported here, LH levels rose modestly on subsequent days during evening sampling compared with morning values, as they also did during vehicle infusion. Consistent nighttime elevations in LH have not previously been noted in agonadal, GnRH-primed juvenile males as young as 21–23 months of age (18), and therefore the present cohort of monkeys may have been more mature than those employed in earlier studies. In any event, nocturnal increases in LH as the first harbinger of the activation of the GnRH pulse generator have been observed in intact as well as gonadectomized male and female monkeys (24–26), normal pubertal children (27), and girls with gonadal dysgenesis (28). It is possible that the dose of hu metastin 45–54 administered (although seemingly high) was not adequate to achieve desensitization of GPR54 in all hypothalamic areas. Alternatively, it is possible that distinct circadian signals to GnRH-induced LH release exist that use pathways that do not include a metastin/GPR54 component. In this regard, it is to be noted that studies of the rat have shown that pretreatment with MK-801 (a NMDA receptor antagonist) fails to abrogate mouse metastin 43–52-induced LH release (29), and in the present experimental primate model, NMDA was able to elicit a LH response during the last day of the hu metastin 45–54 infusion. These findings suggest that glutamate and GPR54 ligands affect GnRH signaling through independent pathways.

Because GnRH can be used to stimulate the reproductive axis in hypogonadotropic states and GnRH receptor agonists and antagonists can be used to perform selective, reversible suppression of the axis in certain cancers, gynecological disorders, and precocious puberty, metastin's down-regulation of GPR54 opens new research opportunities for probing the hypothalamic-pituitary-gonadal axis as well as novel therapeutic possibilities for the treatment of reproductive disorders. Clearly, greater exploration of the physiological and pharmacological features of metastin will be required before this translation to clinical usefulness can occur, but the recent administration of hu metastin to men in a brief infusion (16) holds promise that this peptide can be administered without significant toxicities.

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