Chronic subcutaneous administration of kisspeptin-54 causes testicular degeneration in adult male rats

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Thompson, Emily L., Kevin G. Murphy, Michael Patterson, Gavin A. Bewick, Gordon W. H. Stamp, Annette E. Curtis, Jennifer H. Cooke, Preeti H. Jethwa, Mohammad A. Ghatei, and Stephen R. Bloom. Chronic subcutaneous administration of kisspeptin-54 causes testicular degeneration in adult male rats. Am J Physiol Endocrinol Metab 291:E1074–E1082, 2006. First published June 20, 2006; doi:10.1152/ajpendo.00040.2006.—The kisspeptins are KISS-1 gene-derived peptides that signal through the G protein-coupled receptor-54 (GPR54) and have recently been shown to be critical regulators of reproduction. Acute intracerebroventricular or peripheral administration of kisspeptin stimulates the hypothalamic-pituitary-gonadal (HPG) axis. This effect is thought to be mediated via the hypothalamic gonadotropin-releasing hormone (GnRH) system. Chronic administration of GnRH agonists paradoxically suppresses the HPG axis after an initial agonistic stimulation. We investigated the effects of continuous peripheral kisspeptin administration in male rats by use of Alzet minipumps. Initially we compared the effects of acute subcutaneous administration of kisspeptin-10, -14, and -54 on the HPG axis. Kisspeptin-54 produced the greatest increase in plasma LH and total testosterone at 60 min postinjection and was used in the subsequent continuous administration experiments. Chronic subcutaneous long-term administration of 50 nmol kisspeptin-54/day for 13 days decreased testicular weight. Histological examination showed degeneration of the seminiferous tubules associated with a significant decrease in the circulating levels of the testes-derived hormone, inhibin B. Plasma free and total testosterone were also lower, although these changes did not reach statistical significance. Further studies examined the effects of shorter periods of continuous kisspeptin administration. Subcutaneous administration of 50 nmol kisspeptin-54 for 1 day increased plasma LH and testosterone. This effect was lost after 2 days of administration, suggesting a downregulation of the HPG axis response to kisspeptin following continuous administration. These findings indicate that kisspeptin may provide a novel tool for the manipulation of the HPG axis and spermatogenesis.

metastin; G protein-coupled receptor-54; chronic administration; hypothalamic-pituitary-gonadal axis

The KISS-1 gene codes for a 154-amino acid protein that is cleaved into peptides 54, 14, 13, and 10 amino acids in length (16). These peptides, known as the kisspeptins, are agonists of the G protein-coupled receptor-54 (GPR54) (16, 19, 24, 30). The common COOH-terminal decapeptide shared by all forms, kisspeptin-10, is the minimum sequence necessary for receptor activation (16, 24, 30).

Kisspeptin and GPR54 play a critical role in the central regulation of the hypothalamic-pituitary gonadal (HPG) axis. GPR54 is necessary for normal pubertal development and reproductive function. GPR54 mutations in humans have been shown to cause hypogonadotropic hypogonadism and pubertal delay (7, 37, 38), and GPR54-deficient (GPR54−/−) mice have abnormal sexual development and low circulating gonadotropin concentrations and are incapable of reproducing (9). Acute central or peripheral administration of kisspeptin-54 or kisspeptin-10 potently stimulates the HPG axis (8, 11, 26, 28, 42). This effect appears to be mediated via the hypothalamic gonadotropin-releasing hormone (GnRH) system (18). Peripheral or central administration of kisspeptin activates hypothalamic GnRH neurons (14, 21), the majority of which express GPR54 (14, 15, 22). Kisspeptin-10 stimulates the release of GnRH in vitro and in vivo (22, 42), and the effects of kisspeptin on the HPG axis in vivo can be blocked by GnRH antagonists (11, 14, 21, 39). However, GPR54 is also expressed in the pituitary (16, 24), and kisspeptin has been shown to directly effect the release of gonadotropins in vitro (27, 28). Using different paradigms, other groups have failed to demonstrate a direct pituitary effect on gonadotropin release for kisspeptin (21, 42). The physiological relevance of a direct effect of kisspeptin on the pituitary is therefore currently unknown.

Although the effects of acute administration of kisspeptin on the HPG axis are now well characterized, the effects of continuous chronic kisspeptin administration have not been comprehensively investigated. Twice daily intracerebroventricular (ICV) administration of kisspeptin for 6 days induces vaginal opening in immature females rats (29) and restores vaginal opening in undernourished female rats (5). In male pubertal rats, twice daily ICV administration of kisspeptin for 7 days has been shown not to alter whole hypothalamic/preoptic area GnRH mRNA expression (28). However, the effects of continuous, uninterrupted administration of kisspeptin on the HPG axis have not been studied. Continuous administration of GnRH agonists paradoxically suppresses the HPG axis after a transient stimulation and can cause histological changes in the testes following long-term treatment (44). GnRH agonists are indicated in the treatment of a variety of hormonally responsive clinical disorders, including hormone-dependent tumors and precocious puberty (23, 31). The effects of continuous, chronic, peripheral kisspeptin administration on the HPG axis are therefore of particular interest because kisspeptin may represent a novel tool for HPG axis manipulation in disorders of the reproductive system.
All of the kisspeptins are reported to have a similar affinity and efficacy in vitro at GPR54 (16). However, current data suggest that the different forms of kisspeptin may have different efficiencies at stimulating the HPG axis in vivo (25). We therefore compared the acute effects of subcutaneous (sc) kisspeptin-10, -14, and -54 on the HPG axis in adult male rats. We then examined the effects of long-term continuous 13-day sc kisspeptin-54 administration (50 nmol/day) on the HPG axis in adult male rats. After the unexpected results of this study, we investigated the effect of 1-, 2-, and 3-day continuous sc kisspeptin-54 (50 nmol/day) administration in adult male rats.

METHODS

Materials

Kisspeptin-10, -14, and -54 were synthesized by the Advanced Biotechnology Centre, Imperial College, London, UK.

Animals

Adult male Wistar rats (specific pathogen free, Charles River, UK) weighing 275–325 g were housed in individual cages for the continuous Alzet pump kisspeptin studies and in groups of five for the two acute studies. Animals were maintained under controlled temperature (21–23°C) and light (12:12-h light-dark cycle, lights on at 0700) conditions with ad libitum access to food (RM1 Diet, SDS, Witham, UK) and water. All animal procedures were conducted under the British Home Office Animals Scientific Procedures Act of 1986 (Project Licence 70/5516) and in accordance with accepted standards of the local ethics review committee.

Acute Peripheral sc Kisspeptin-10, -14, and -54 on Plasma LH and Total Testosterone

To determine which form of kisspeptin to use in the continuous Alzet pump sc kisspeptin studies, the effects of kisspeptin-10, kisspeptin-14, and kisspeptin-54 on the HPG axis were compared. Rats received a single sc injection of 0.1 ml of 0.9% saline daily for 3 days to acclimatize them to the injection procedure, and they also underwent sham decapitation for 2 days before the study day. On the following day, rats (n = 5–7/group) were sc injected with 0.1 ml of 0.9% saline, kisspeptin-10, kisspeptin-14, or kisspeptin-54 at 0.1, 0.3, 1.0, and 50 nmol. At 60 min following injection, rats were decapitated, and trunk blood was collected into lithium-heparin tubes containing 0.6 mg of aprotinin (Bayer). Plasma was separated by centrifugation, frozen on dry ice, and stored at −20°C until measurement of LH and total testosterone.

Determination of Alzet Pump Kisspeptin Viability: Acute sc Injection of Freshly Prepared Kisspeptin-54 or Kisspeptin-54 Incubated at 37°C for 14 Days

To investigate the stability of kisspeptin-54 in the Alzet osmotic minipumps over 14 days at 37°C, an Alzet pump (model 2002) was filled with kisspeptin-54 for 4.16 nmol/µl, the same concentration as that intended to be used in the chronic 13-day in vivo study. The Alzet pump was then incubated at 37°C for 14 days. After this 14-day incubation, adult male rats (n = 5/group), acclimatized to the injection procedure as described above, received a sc injection of 0.1 ml of 0.9% saline, 1 nmol freshly prepared kisspeptin-54, or 1 nmol kisspeptin-54 from the incubated Alzet pump. At 60 min following injection, rats were decapitated and trunk blood collected and stored as above until measurement of LH and total testosterone.

Alzet Miniosmotic Pump Insertion and Peptide Delivery

Alzet miniosmotic pumps, model 2001D (1-day treatment), model 2001 (2- and 3-day treatment), and model 2002 (13-day treatment), were filled with either 0.9% saline (control) or kisspeptin-54 (dissolved in 0.9% saline to deliver at a rate of 50 nmol/day) under sterile conditions. The Alzet pumps were then primed in vials containing 0.9% saline at 37°C overnight (models 2001 and 2002) or for 3 h (model 2001D) before implantation so that they would deliver their contents immediately on implantation. The Alzet model 2001D infuses solutions at a rate of 8.0 ± 1.0 µl/h for 1 day; model 2001 infuses solutions at a rate of 1.0 ± 0.15 µl/h for 7 days; and model 2002 infuses solutions at a rate of 0.5 ± 0.1 µl/h for 14 days.

Long-Term Study

Continuous chronic sc kisspeptin-54 administration (50 nmol/day for 13 days). The rats were randomized into two groups (n = 10/group). On day 0, under deep inhalation anesthesia (halothane; Concord Pharmaceuticals, Dunmow, UK), the rats were implanted subcutaneously in the interscapular region with the preloaded, primed pumps, containing either 0.9% saline or kisspeptin-54. Prophylactic antibiotics flucloxacillin (37.5 mg/kg) and amoxicillin (37.5 mg/kg) were administered before surgery. A small incision was made between the skin and the scapulae with a sterile blade. Subcutaneous tissue was spread using a hemostat to create a pocket that was ~50% greater than the diameter of the pump. The pump was inserted into the pocket with the flow moderator facing away from the incision, and the skin incision was closed with wound clips (Vet Tech Solutions, Cheshire, UK). Rats were allowed to recover before being returned to their home cage.

Long-term study procedure. From day 1, body weight and food intake were measured each morning between 0900 and 1000. Behavior was observed throughout the study. Rats were killed by decapitation on day 13 of the study between 0900 and 1100. Rats were killed on day 13 to guarantee peptide delivery continued for the entire study. Trunk blood was collected into lithium-heparin tubes containing 0.6 mg of aprotinin (Bayer). Plasma was separated by centrifugation, frozen on dry ice, and stored at −20°C until measurement of LH, FSH, total testosterone, free testosterone, inhibin B, and activin A. The left testis (n = 10), left epididymis (n = 5), and paired seminal vesicles (empty; n = 5) were immediately dissected and weighed. In addition, the Alzet pumps were removed and examined to verify that they had delivered their contents as expected.

Investigation of Effects of Long-Term Continuous Chronic sc Kisspeptin Administration (50 nmol/day for 13 days) on Testis Histology: Histological Procedures

After careful removal to avoid mechanical trauma, the left testis from the 13-day-treated rats were perforated with a fine-gauge needle, immersion fixed in Bouin’s fixative for 24 h, and subsequently stored in 70% ethanol until processing and embedding in paraffin. After embedding, testes were serially sectioned at 4 μm and stained with hematoxylin and eosin (H&E) for histology (n = 5/group). The tissue sections close to the largest cross-sectional area of the organs were used in the microscopic assessment. Between 53 and 180 seminiferous tubules were examined per animal, and the criteria used for histological examination followed the principles described in standard methods of rodent testicular analysis (35).
Concord Pharmaceuticals), the rats were implanted sc in the interscapular region with the preloaded, primed pumps, containing either 0.9% saline or kisspeptin-54 (Alzet models used are detailed above). The surgery was performed as described above for the chronic continuous 13-day administration study.

Short-term study procedure. From day −1, body weight and food intake were measured each morning between 0900 and 1000. Behavior was observed throughout the study. Rats were killed by decapitation on day 1, 2, or 3 of the study between 0900 and 1100. Trunk blood was collected as above and stored at −20°C until measurement of LH, FSH, and free testosterone. In addition, the Alzet pumps were removed from all groups and examined to verify that they had delivered their contents as expected.

Hormone Assays

LH levels in plasma were assayed using reagents and methods provided by the National Institute of Diabetes and Digestive and Kidney Diseases and the National Hormone and Pituitary Program (Dr. A. Parlow, Harbor University, Los Angeles Medical Center, Los Angeles, CA), as previously described (3). The intra- and interassay coefficients of variation were 8.2 and 13.6%, respectively. Plasma FSH was measured by commercial immunoradiometric assay (IDS, Boldon, UK) and total plasma testosterone by commercial Coat-a-Count assay kit (EURO/DPC, Caernarfon, UK). Free testosterone in plasma was measured by EIA (Diagnostic Systems Laboratories, Oxfordshire, UK) (12). The intra- and interassay coefficients of variation for the commercial assays were <10%.

Statistical Analysis

Results are shown as mean values ± SE. Data from plasma hormone measurements following acute sc administration of different doses and forms of kisspeptin were compared by analysis of variance (ANOVA) with post hoc Tukey adjustment (Systat, Evanston, IL). Plasma hormone measurements following continuous chronic sc administration of kisspeptin-54 for 13 days were compared by unpaired t-test between saline- and kisspeptin-54-treated groups. Plasma hormone measurements following continuous sc administration of kisspeptin-54 for 1, 2, or 3 days were compared by ANOVA with post hoc Tukey adjustment. Data from plasma hormone measurements following sc administration of fresh and Alzet pump-incubated kisspeptin were compared by ANOVA with post hoc Tukey adjustment. In all cases, P < 0.05 was considered to be statistically significant.

RESULTS

Effect of Acute sc Administration of Kisspeptin-10, -14, and -54 on Plasma LH and Total Testosterone

Kisspeptin-54 produced the largest increase in plasma LH. Acute sc injection of 1 and 50 nmol kisspeptin-54 significantly increased plasma LH at 60 min postinjection, ~9- and 12-fold respectively, compared with saline control. A dose of 0.3 nmol kisspeptin-54 also increased plasma LH, but this change did not achieve statistical significance. Acute sc injection of the highest dose administered of kisspeptin-10 and kisspeptin-14, 50 nmol, also produced a trend toward increased plasma LH at

Fig. 1. Effect of acute sc administration of saline, kisspeptin (KP)-10, kisspeptin-14, or kisspeptin-54 (0.1, 0.3, 1, and 50 nmol) on plasma levels of LH (A) and total testosterone (B) in male Wistar rats at 60 min postinjection. In A and B, significance is indicated by ***P < 0.001 vs. saline control; n = 5–7/group.

Fig. 2. Stability of kisspeptin-54 incubated for 14 days at 37°C. Effect of acute sc administration of 1 nmol freshly prepared kisspeptin-54 and 1 nmol kisspeptin-54 incubated for 37°C for 14 days in an Alzet pump on plasma levels of LH (A) and total testosterone (B) in adult male Wistar rats at 60 min postinjection. Significance is indicated by *P < 0.05, freshly prepared kisspeptin or Alzet pump kisspeptin vs. saline control; n = 5/group.
60 min postinjection, but these changes did not achieve statistical significance (Fig. 1A).

Kisspeptin-54 produced the largest increase in plasma total testosterone. Acute sc injection of 0.3, 1, and 50 nmol kisspeptin-54 significantly increased plasma total testosterone at 60 min postinjection. The lowest dose of kisspeptin-54 administered, 0.1 nmol, also increased plasma total testosterone, but this change did not reach statistical significance. A 50-nmol dose of kisspeptin-14 also produced a significant increase in plasma total testosterone, with the lower dose, 1 nmol kisspeptin-14, producing a trend toward increased plasma total testosterone, which did not reach statistical significance. The testos-

Table 1. Absolute (g) and relative (g/100 g) weights of the reproductive organs of adult male rats following chronic, continuous, long-term (13-day) sc treatment with saline or 50 nmol/day kisspeptin-54

<table>
<thead>
<tr>
<th></th>
<th>Testis (L)</th>
<th>Epididymis (L)</th>
<th>Seminal Vesicles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Kisspeptin</td>
<td>Control</td>
</tr>
<tr>
<td>g</td>
<td>1.92±0.04</td>
<td>1.53±0.06†</td>
<td>0.29±0.03</td>
</tr>
<tr>
<td>g/100 g</td>
<td>0.52±0.01</td>
<td>0.41±0.02*</td>
<td>0.13±0.01</td>
</tr>
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</table>

Data are reported as means ± SE; n = 5–10/group. Control, saline-treated controls; kisspeptin, 50 nmol/day kisspeptin-54-treated group. Testis and epididymis are left (L) only. Seminal vesicles are paired and empty. Significance is indicated by *P < 0.001, †P < 0.0001 vs. saline control.
terone response following injection of kisspeptin-10 was limited, with 50 nmol kisspeptin-10 producing a nonsignificant increase in plasma total testosterone (Fig. 1B).

Stability of Alzet Pump Kisspeptin-54: Effect of Acute sc Administration of Freshly Prepared Kisspeptin-54 or Kisspeptin-54 Incubated at 37°C for 14 Days on Plasma LH and Total Testosterone

Acute sc administration of 1 nmol freshly prepared kisspeptin-54 and 1 nmol kisspeptin-54 that had been incubated at 37°C in an Alzet pump for 14 days potently increased plasma LH and total testosterone to a similar extent at 60 min postinjection (Fig. 2). There was no significant difference between the effect of freshly prepared kisspeptin-54 and kisspeptin-54 incubated in an Alzet pump for 14 days at 37°C on plasma LH or total testosterone.

Long-Term Study: Effect of Continuous Chronic sc Administration of 50 nmol/day Kisspeptin-54 for 13 Days on the HPG Axis

Food intake, body weight, and behavior. Food consumption and body weight gain were equivalent in all kisspeptin-treated and control groups, suggesting that the animals were healthy throughout the study period. No adverse behaviors were observed in any of the treatment groups at any point during the study.

Circulating hormones. Chronic sc administration of 50 nmol/day kisspeptin-54 led to significantly decreased plasma inhibin B. Plasma activin A was not altered following chronic sc administration of 50 nmol/day kisspeptin-54. Chronic sc administration of 50 nmol/day kisspeptin-54 produced a trend toward decreased total testosterone and free testosterone, although this did not reach statistical significance. Interestingly, the chronic sc administration of 50 nmol/day kisspeptin-54 for 13 days had no significant effect on plasma LH and FSH (Fig. 3).

Reproductive Organs

The absolute and relative weights of the left testes were significantly reduced following long-term, 13-day, continuous, chronic kisspeptin-54 administration compared with saline controls. The left epididymis and paired seminal vesicles (empty) weights were similar between the two groups (Table 1). All saline-treated animals had normal testicular histology with no seminiferous cell degeneration observed (Figs. 4A and 5A). Chronic administration of 50 nmol/day kisspeptin-54 for 13 days led to significant focal damage of the seminiferous tubules. Individual tubules showed varying degrees of germ cell maturation arrest, formation of multinucleated spermatid giant cells formed from round spermatids, and sloughing and death of germ cells with complete focal loss of germ cells and degeneration of residual Sertoli cells. In areas, this was accompanied by mononuclear inflammatory cell infiltration and interstitial edema. There were no measurable differences in Leydig cell morphology (Figs. 4B and 5, B and C). Interestingly, the effect of chronic kisspeptin treatment on testicular histology varied between animals (Table 2).

Short-Term Study: Effect of Continuous sc Kisspeptin-54 Administration (50 nmol/day for 1, 2, or 3 days) on Hormones of the HPG Axis

Continuous sc administration of 50 nmol/day kisspeptin-54 for 1 day significantly increased plasma LH almost threefold compared with saline-treated controls. This increase in plasma LH was lost after 2 days of continuous kisspeptin treatment and remained indistinguishable from saline controls after 3 days of treatment (Fig. 6A). Interestingly, the chronic sc administration of 50 nmol/day kisspeptin-54 for 1, 2, or 3 days had no significant effect on plasma FSH (Fig. 6B). Continuous sc administration of 50 nmol/day kisspeptin-54 led to an acute, significant fourfold increase in plasma free testosterone compared with saline-treated controls after 1 day of kisspeptin-54
administration. This stimulatory effect was lost by 2 days of continuous treatment with kisspeptin (Fig. 6C).

DISCUSSION

The kisspeptins have recently been identified as key regulators of the HPG axis. Although the acute effects of central and peripherally administered kisspeptin have been explored, the effects of long-term administration have been less thoroughly investigated. We have directly compared the acute bioefficacies of different forms of kisspeptin when administered sc and demonstrated that sc administration of 50 nmol kisspeptin-54/day for 13 days to adult male rats leads to a significant reduction in testicular weight, accompanied by testicular degeneration and a significant decrease in circulating inhibin B levels. There was also a trend toward decreased plasma total and free testosterone. However, these changes in testicular hormones were not associated with a significant change in plasma gonadotropin levels. Investigating the effects of shorter periods of continuous kisspeptin-54 administration demonstrated an initial increase in plasma LH and free testosterone after 1 day of treatment. This effect was lost after 2 days of kisspeptin administration.

Our initial acute study investigating the effects of kisspeptin-10, -14, and -54 on the HPG axis suggests that these forms have different bioefficacies when administered sc to rats. Kisspeptin-10, -14, and -54 have similar affinities and efficacies at GPR54 in vitro (16). Moreover, in vivo ICV administration of kisspeptin-10 or kisspeptin-54 appears to stimulate gonadotropin release to a similar magnitude (11). However, in the present study, we compared the effects of sc kisspeptin-10, -14, and -54 on plasma LH and total testosterone at 60 min postinjection and found the longest form, kisspeptin-54, to have the greatest effect on plasma LH and total testosterone. We have shown that the lowest effective sc dose of kisspeptin-54 to produce a significant increase in plasma LH at 60 min postinjection was 1 nmol, with a dose of 0.3 nmol kisspeptin-54 significantly increasing total testosterone at 60 min, indicative of an earlier LH response at this dose. Subcutaneous administration of the shorter forms, kisspeptin-10 and -14, did not significantly raise plasma LH at this time point, but kisspeptin-14 did significantly increase plasma total testosterone levels, suggesting that this shorter form of kisspeptin did increase plasma LH at an earlier time point. Kisspeptin-10 is the shortest active form of kisspeptin and consists of the 10 amino acid-amidated carboxyl terminal sequence vital for receptor interaction (30). However, at 60 min postinjection, kisspeptin-10 at doses from 0.1 to 50 nmol did not significantly change plasma LH or total testosterone. Shorter forms of kisspeptin may be cleared or inactivated more rapidly than longer forms when administered peripherally. As subcutaneous full-length kisspeptin-54 appeared to produce the most persistent effect on circulating LH and total testosterone, it was decided to use this form of kisspeptin in the subsequent continuous administration studies.

To verify that kisspeptin-54 would remain relatively stable for 14 days at 37°C, an Alzet pump was filled with kisspeptin-54 and incubated at 37°C for 14 days. Importantly, acute

Fig. 5. Cross section of individual testicular tubules assessed by light microscopy on H&E-stained sections from a saline-treated control (A) and animals treated with sc administration of 50 nmol/day kisspeptin-54 for 13 days (B and C). Original magnifications ×400; scale bar, 25 μm. A: representative section from a control animal shows a tubule with normal maturation of germ cells to spermatozoa (arrow). B and C: representative sections from animals treated with chronic 13-day kisspeptin administration, showing tubules with severe necrotic degeneration of germinal and Sertoli cells (*), leaving residual eosinophilic debris (e, arrow) (B), and degeneration characterized by multinucleated giant cells (m, arrow) (C). C also shows interstitial edema (i, arrow).
administration of 1 nmol incubated kisspeptin caused an increase in plasma LH and total testosterone of a similar magnitude to freshly prepared kisspeptin-54. This 1-nmol dose was chosen to test for kisspeptin-54 activity following pump incubation because it was the lowest dose to significantly increase plasma LH in the acute kisspeptin-54 dose-response study. The results suggested that kisspeptin-54 would remain stable for the duration of the continuous administration studies.

Chronic, long-term (13-day), continuous sc administration of 50 nmol kisspeptin-54/day to adult male rats has suppressive effects on various parameters of the HPG axis. The most striking effects of chronic kisspeptin administration were on the testes. There was a significant decrease in testis weight and a significant degeneration of the seminiferous tubules, with loss of both germ and Sertoli cells. The decrease in testicular weight and testicular degeneration was accompanied by a significant decrease in circulating inhibin B. This testis-derived hormone has been proposed as a sensitive endocrine marker reflecting the state of spermatogenesis. Inhibin B levels are well correlated with Sertoli cell number (34, 40), with low levels reflecting a disturbance of spermatogenesis (32). Testicular degeneration similar to that found in our study has been observed following continuous administration of GnRH agonists (33, 43, 44). The continuous administration of GnRH agonists initially evokes an agonist phase, which lasts several days or weeks, followed by the desensitization and suppression of the HPG axis (6). The major mechanism is thought to be the downregulation of pituitary GnRH receptors, leading to a suppression of circulating gonadotropins and sex steroids (1, 17, 41). As the effects of kisspeptin on the HPG axis are thought to be mediated via hypothalamic GnRH (25), a decrease in gonadotropins might be expected following the continuous administration of kisspeptin-54. Interestingly, in the present study, the continuous administration of 50 nmol kisspeptin-54/day for 13 days did not significantly change circulating LH or FSH concentrations. Acute kisspeptin potently stimulates LH and FSH release. To verify that there was an early stimulatory effect on the HPG axis after continuous kisspeptin-54 administration, we examined the effects of short-term continuous sc administration of kisspeptin-54 at 50 nmol/day for 1, 2, or 3 days on circulating gonadotropins and free testosterone levels. We have shown that, in the adult male rat, sc administration of 50 nmol/day kisspeptin-54 for 1 day leads to an initial stimulatory effect on plasma LH and testosterone, which is abolished by 2 days of continuous treatment. This suggests that continuous kisspeptin administration leads to desensitization of the HPG axis response to kisspeptin administration. During the preparation of this paper, Seminara et al. (36) published data showing that continuous intravenous kisspeptin-10 administration to agonadal juvenile male monkeys leads to plasma LH levels similar to control values by 12 h following an initial acute agonistic flair. The authors suggest that the desensitization of the HPG axis to kisspeptin in the primate is via desensitization of hypothalamic GPR54 (36). Sustained stimulation of GPCRs typically causes receptor desensitization that is mediated by phosphorylation, often within the COOH-terminal tail of the receptor, which leads to arrestin binding. This prevents the receptor from binding and activating G proteins and can also target it for internalization via clathrin-mediated endocytosis, causing receptor loss from the cell surface (10). As a typical GPCR, GPR54 desensitization is a likely mechanism by which the HPG axis response to kisspeptin is downregulated following continuous kisspeptin

Table 2. Effect of chronic, continuous, long-term (13-day) sc treatment with 50 nmol/day kisspeptin-54 on seminiferous tubule histology

<table>
<thead>
<tr>
<th>Kisspeptin-Treated Animal Number</th>
<th>Seminiferous Tubules Showing Degeneration</th>
<th>Seminiferous Tubules Showing Degeneration with Mononuclear Cell Infiltration</th>
<th>Normal Seminiferous Tubules</th>
</tr>
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<tr>
<td>1</td>
<td>28.9</td>
<td>33.3</td>
<td>37.8</td>
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<td>3</td>
<td>18.6</td>
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<td>37.8</td>
</tr>
<tr>
<td>9</td>
<td>43.4</td>
<td>22.7</td>
<td>34.0</td>
</tr>
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</table>

Values are percentages. No saline-treated animals had abnormal testicular histology.

Fig. 6. Effect of continuous, short-term (1-, 2-, and 3-day) sc administration of saline (gray bars) or 50 nmol/day kisspeptin-54 (cross-hatched bars) in male Wistar rats on plasma levels of LH (A), FSH (B), and free testosterone (C). Significance is indicated by **P < 0.01, ***P < 0.001 vs. saline control; n = 9–10/group.
administration. Further studies are needed to investigate the exact mechanism by which this might occur.

In the present study, although continuous sc administration of kisspeptin-54 appears to have led to a desensitization of the HPG axis to kisspeptin, it did not lead to a suppression of circulating gonadotropins. It is possible that, although basal circulating gonadotropin levels are unchanged, there is a change in their pulsatile release that is responsible for the downstream effects on testicular histology. Another possibility is a direct testicular effect. After chronic GnRH administration, there is evidence that testicular degeneration occurs before a significant suppression of circulating gonadotropins (43). Some reports of the effects of GnRH agonists also suggest an extrapituitary direct inhibitory action on the gonads (2, 13, 43).

GPR54 has been reported to be expressed in the testes (9, 16, 30). The effects of chronic administration of kisspeptin-54 on the testes may therefore be direct. It is possible that the testicular degeneration seen after chronic kisspeptin administration may represent changes in testicular blood flow. Short-term decreases in flow result in apoptosis among spermatogonia and early spermatocytes (4), with moderate, long-term reductions in blood flow leading to focal damage of the seminiferous tubules and disturbance of spermatogenesis (20), and may explain our current findings. It would be interesting to look at the effects of continuous long-term kisspeptin administration in hypophysectomized rats and also the effects on the microcirculation of the testes. Investigating the effects of continuous administration of kisspeptin directly into the hypothalamus might also help determine whether the testicular degeneration is centrally or peripherally mediated.

In summary, these findings suggest that kisspeptin repre-


