RESEARCH PAPER

Kisspeptin-54 at high doses acutely induces testicular degeneration in adult male rats via central mechanisms

EL Thompson[1], V Amber[1], GWH Stamp[2], M Patterson[1], AE Curtis[1], JH Cooke[1], GF Appleby[1], WS Dhillo[1], MA Ghatei[1], SR Bloom[1] and KG Murphy[1]

[1]Department of Investigative Medicine, Hammersmith Hospital, Imperial College London, London, UK, and [2]Department of Histopathology, Hammersmith Hospital, Imperial College London, London, UK

Background and purpose: The kisspeptins are critical regulators of reproduction and a therapeutic target for reproductive disease. Intracerebroventricular (i.c.v.) or peripheral injection of kisspeptin potently stimulates the hypothalamic-pituitary gonadal (HPG) axis via gonadotrophin-releasing hormone (GnRH). However, little is known regarding the effects of kisspeptin administration on testicular function. We investigated the mechanism(s) of kisspeptin-induced testicular degeneration in the rat.

Experimental approach: Kisspeptin-54 (50 nmol·day^{-1}) was continuously administered subcutaneously (6 h to 3 days) to male Wistar rats and reproductive hormones and testicular histology analysed. We also investigated the effects of a single subcutaneous injection of 0.5, 5 or 50 nmol kisspeptin-54. In order to determine whether the testicular degeneration observed is peripherally or centrally mediated, we investigated effects of i.c.v. injections of 5 nmol kisspeptin-54 and pre-administered a GnRH-receptor antagonist (cetrorelix) to rats peripherally treated with kisspeptin-54.

Key results: Continuous subcutaneous administration of kisspeptin-54 caused testicular degeneration after only 12 h, when gonadotrophins were still markedly raised, suggesting that the degeneration is independent of the desensitization of the HPG axis to kisspeptin-54. Furthermore, a single subcutaneous injection of kisspeptin-54 caused dose-dependent testicular degeneration. Continuous kisspeptin-54 administration is thus not required to cause testicular degeneration. Pretreatment with cetrorelix blocked kisspeptin-induced testicular degeneration, and a single i.c.v. injection of kisspeptin-54 caused testicular degeneration, suggesting it is GnRH-mediated.

Conclusions and implications: Kisspeptin-induced testicular degeneration appears to be centrally mediated, and result from acute hyper-stimulation of the HPG axis. Doses must be carefully considered if kisspeptin is to be used therapeutically.
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Introduction

The *KiSS-1*/G protein-coupled receptor-54 (GPR54) system has recently been identified as a critical component of the hypothalamic-pituitary gonadal (HPG) axis. Mice and humans lacking functional GPR54 or *KiSS-1* genes do not undergo pubertal development and are infertile (Funes *et al.*, 2003; de Roux *et al.*, 2003; Seminara *et al.*, 2003; Semple *et al.*, 2005; d'Anglemont de Tassigny *et al.*, 2007; Lapatto *et al.*, 2007). The *KiSS-1* gene encodes a 145 amino acid protein (Muir *et al.*, 2001; Ohtaki *et al.*, 2001) that is cleaved into kisspeptin-54, corresponding to residues 68–121 of the full length *KiSS-1* product (Ohtaki *et al.*, 2001). Shorter *KiSS-1* derived peptides, 14, 13 and 10 amino acids in length have also been identified (Kotani *et al.*, 2001; Bilban *et al.*, 2004). All of the kisspeptins are reported to have a similar affinity and efficacy *in vitro* at GPR54 (Kotani *et al.*, 2001). However, kisspeptin-54 is likely to be the most effective form of kisspeptin *in vivo* (Thompson *et al.*, 2006).

The stimulatory effects of kisspeptin on the HPG axis are now well characterized. Acute intracerebroventricular (i.c.v.) or peripheral administration of kisspeptin to rodents or primates, and peripheral administration to humans, stimulates the HPG axis (Tena-Sempere, 2006; Mead *et al.*, 2007a). This effect is likely to be mediated primarily via hypothalamic gonadotrophin-releasing hormone (GnRH) (Gottsch *et al.*, 2004; Irwig *et al.*, 2004; Matsui *et al.*, 2004; Thompson *et al.*, 2004; Kinoshita *et al.*, 2005; Messager *et al.*, 2005; Shahab *et al.*, 2005). GPR54 is expressed in the pituitary (Kotani *et al.*, 2001; Muir *et al.*, 2001; Kinoshita *et al.*, 2005), and *in vitro* evidence suggests that kisspeptin can have a direct effect on gonadotrophin release from cultured rat pituitary cells (Navarro *et al.*, 2005a,b). However, other studies have shown no direct effect of kisspeptin on gonadotrophin secretion from cultured rat pituitary cells or pituitary fragments *in vitro* (Matsui *et al.*, 2004; Thompson *et al.*, 2004). In addition, it has recently been shown that hypothalamic-pituitary disconnected ewes maintained with pulsatile GnRH do not respond to exogenous intravenous administration of kisspeptin (Smith *et al.*, 2007), suggesting that direct effects of kisspeptin on the pituitary are unlikely to play a major role in kisspeptin-induced stimulation of the HPG axis *in vivo*.

The kisspeptin-GPR54 system is a putative target for drugs to treat reproductive disorders and manipulate the reproductive axis. Administration of kisspeptin to humans potently stimulates the HPG axis (Dhillo *et al.*, 2005; Dhillo *et al.*, 2007). The kisspeptins may thus have utility in initiating puberty in adolescents with delayed puberty and in the treatment of adult infertility. Conversely, GPR54 antagonists may be useful in the treatment of precocious puberty and as contraceptive agents. Interestingly, continuous peripheral administration of kisspeptins to monkeys and rodents results in desensitization of the HPG axis to their effects (Seminara *et al.*, 2006; Thompson *et al.*, 2006; Ramaswamy *et al.*, 2007). Furthermore, the chronic long-term (13 days) continuous s.c. [subcutaneous(ly)] administration of 50 nmol kisspeptin-54 per day to adult rats leads to significant degeneration of the testes, with loss of both germ and Sertoli cells (Thompson *et al.*, 2006). The mechanism by which chronic kisspeptin-54 causes testicular degeneration is unknown. Understanding the mechanism(s) of kisspeptin-54-induced testicular degeneration is essential if the kisspeptins are to be used to manipulate the reproductive axis in humans.

In the present study we investigated the mechanism(s) of kisspeptin-54-induced testicular degeneration in the adult rat. We performed a time course analysis of the effects of continuous s.c. kisspeptin-54 administration on testicular histology and plasma reproductive hormone levels. Surprisingly, continuous s.c. kisspeptin-54 administration led to testicular degeneration as early as 12 h after commencing continuous administration. We therefore investigated the effects of a single s.c. injection of high-dose kisspeptin-54 (50 nmol) on testicular histology and compared them with the effects of a high-dose s.c. injection of GnRH (50 nmol). In order to determine whether the testicular degeneration observed following both continuous and single s.c. high-dose kisspeptin-54 administration is peripherally or centrally mediated, we investigated the effect of a single i.c.v. injection of kisspeptin-54 on testicular histology. We also pre-

administered a GnRH-receptor (GnRH-R) antagonist, cetrorelix, to rats peripherally treated with kisspeptin-54 to further clarify whether the effects of kisspeptin-54 on the testes are centrally mediated. Finally, we investigated whether the testes show histological recovery from kisspeptin-54-induced testicular degeneration, and whether there were any prolonged effects on plasma hormone levels. Taken together, the results suggest that kisspeptin-induced testicular degeneration is centrally mediated and results from acute hyper-stimulation of the HPG axis. The present findings are potentially of clinical significance if the kisspeptins are to be used as a treatment in reproductive disorders and suggest the therapeutic doses of kisspeptins will need to be chosen with care.

Methods

Animals
All animal procedures were conducted under the British Home Office Animals Scientific Procedures Act 1986 (Project License 70/5516) and in accordance with accepted standards of the local ethical review committee. Adult male Wistar rats (specific pathogen free, Charles River) weighing 275–325 g were housed in individual cages for the continuous Alzet® pump kisspeptin-54 studies and the i.c.v. study, and in groups of five for the peripheral single injection studies. Animals were maintained under controlled temperature (21–23°C) and light conditions (12:12 h light/dark cycle, lights on at 0700 h) with *ad libitum* access to food (RM1 diet, SDS UK Ltd.) and water.

Alzet® mini-osmotic pump insertion and peptide delivery
Alzet® mini-osmotic pumps, model 2001D (6 h, 12 h and 1 day treatment), model 2001 (2 and 3 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^{-1}) under sterile conditions, following the manufacturers instructions. The Alzet® pumps were then primed in vials containing 0.9% saline at 37°C overnight (model 2001), or for 3 h (model 2001D), before implantation so that they would deliver their contents immediately upon implantation. The Alzet® model 2001D infuses solutions at a rate of 8.0 μL·h^{-1} ($\pm$1.0 μL·h^{-1}) for 1 day, and model 2001 infuses solutions at a rate of 1.0 μL·h^{-1} ($\pm$0.15 μL·h^{-1}) for 7 days.

Study A: Effect of continuous s.c. kisspeptin-54 administration (50 nmol·day^{-1}) for 1, 2 or 3 days on testicular histology and plasma inhibin B
We investigated the time course of effects of continuous administration of kisspeptin-54 on testicular degeneration and on plasma inhibin B levels. Rats were randomized into six groups (*n* = 8 per group). On day 0, under deep inhalation anaesthesia (halothane, Concord Pharmaceuticals Ltd) the rats were implanted s.c. in the interscapular region with the preloaded, primed pumps, containing either 0.9% saline or kisspeptin-54 (to deliver at a rate of 50 nmol·day^{-1}) (Alzet® models used are detailed above). The surgery was performed as previously described (Thompson *et al.*, 2006).

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From day –1, body weight and food intake were measured each morning between 0900 h and 1000 h. Behaviour was observed throughout the study. Rats were killed by decapitation on days 1, 2 or 3 of the study between 0900 h and 1100 h. Trunk blood was collected into lithium-heparin tubes containing 0.6 mg of aprotinin (Bayer, Haywards Heath, UK). Plasma was separated by centrifugation, frozen on dry ice and stored at –20°C until measurement of inhibin B.

Histological examination

Following careful removal to avoid mechanical trauma, the left testis from the 1, 2 and 3 day treated rats ($n = 8$ per group) was 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. Following embedding, testes were serially sectioned at the longitudinal plane at 4 μm and stained with haematoxylin and eosin (H&E) following standard techniques (Drury and Wallington, 1967) for histology. The tissue sections closest to the largest cross-sectional area of the organs were used in the microscopic assessment (light microscope: Nikon Eclipse 80i; digital camera: Nikon DXM1200F). An average number of 250 seminiferous tubules were examined per animal, and the criteria used for histological examination followed the principles described in standard methods of rodent testicular analysis (Russell *et al.*, 1990). Testicular degeneration was qualitatively assessed and quantitatively scored. The percentage of seminiferous tubules degenerated per section was scored as in previously published studies (Leon *et al.*, 1987; Kerr and Sharpe, 1989). In addition, the Alzet® pumps were removed from all groups and examined to verify delivery of their contents.

Study B: (i) Investigating the earliest time point at which testicular degeneration is present following continuous s.c. kisspeptin-54 administration, and (ii) examining the hormonal profile at these early continuous administration time points

The results from Study A demonstrated that continuous s.c. kisspeptin-54 treatment led to testicular degeneration following only 1 day of continuous administration, which was the earliest time point examined. We therefore investigated the effects of shorter-term continuous kisspeptin-54 treatment (6, 12, and 24 h continuous kisspeptin-54 administration, to deliver at a rate of 50 nmol·24 h^{-1}) on testicular degeneration and plasma luteinising hormone (LH), follicle-stimulating hormone (FSH) and testosterone. Rats were randomized into six groups ($n = 5$ per group). At time zero ($t = 0$) under deep inhalation anaesthesia (halothane, Concord Pharmaceuticals Ltd) the rats were implanted s.c. in the interscapular region with the preloaded, primed pumps, containing either 0.9% saline or kisspeptin-54 (Alzet® model 2001D, detailed above). Rats received continuous s.c. administration of saline or kisspeptin-54 and were killed by decapitation 6, 12 or 24 h after Alzet® pump insertion. Trunk blood was collected and stored as described above until measurement of LH, FSH and free testosterone. The left testes ($n = 5$ per group) were immediately dissected, weighed and prepared for histological analysis and assessed as described above. In addition, the Alzet® pumps were removed from all groups and examined to verify content delivery.

(iii) Investigating the specificity of kisspeptin-54-induced testicular degeneration

The possibility that the damaging effects of kisspeptin-54 on the testes could be due to contaminants in the kisspeptin-54 preparation and/or non-specific tissue degenerative effects was investigated. Aliquots of the same batch of kisspeptin-54 used in these studies were sent for pyrogen analysis by the Limulus Amoebocyte Lysate assay test (Associates of Cape Cod, Liverpool, UK). In addition, following continuous s.c. administration of saline or 50 nmol kisspeptin-54 for 24 h the vas deferens, prostate, epididymis, liver, heart, lung, small intestine and spleen were quickly removed, immersion fixed in buffered 10% formalin and subsequently stored in 70% ethanol until processing and embedding in paraffin. Following embedding, tissues were serially sectioned at 4 μm and stained with H&E following standard techniques (Drury and Wallington, 1967), for histological analysis ($n = 5$ per group).

(iv) Investigating the effect of a single s.c. injection of kisspeptin-54 on testicular degeneration

Following the results from Study A showing that short-term s.c. continuous kisspeptin-54 administration led to testicular degeneration in adult male rats, the effects of a single s.c. 50 nmol kisspeptin-54 injection on testicular degeneration was investigated to determine whether the continuous administration of kisspeptin-54 is necessary for these degenerative effects. The 50 nmol dose of kisspeptin-54 was chosen as this was the dose given over 24 h in the continuous administration studies, and allows the comparison of the testes at 24 h following a single injection with those from the 24 h continuously treated group. Rats were randomized into two groups ($n = 5$ per group). At $t = 0$, rats received a single s.c. injection of either 0.9% saline or 50 nmol kisspeptin-54 and were killed by decapitation 24 h following injection. The left testes ($n = 5$ per group) were immediately dissected, weighed and prepared for histological analysis and assessed as described above.

Study C: (i) Dose response of single s.c. injections of kisspeptin-54 on testicular degeneration and comparison with a single s.c. injection of GnRH

We performed a dose–response study of the effects of acute s.c. administration of kisspeptin-54 on the testes. Evidence suggests that kisspeptin acts upstream of hypothalamic GnRH (Gottsch *et al.*, 2004; Irwig *et al.*, 2004; Matsui *et al.*, 2004; Thompson *et al.*, 2004; Kinoshita *et al.*, 2005; Messager *et al.*, 2005; Shahab *et al.*, 2005). Continuous administration of GnRH agonists can also lead to testicular degeneration (Sandow *et al.*, 1978; Rivier *et al.*, 1979; Lefebvre *et al.*, 1984; Rajfer *et al.*, 1984; van Kroonenburgh *et al.*, 1986; Ward *et al.*, 1989; Zheng and Fulu, 2006). However, the effects of acute administration of GnRH are less characterized. We thus also compared the effects of an equal high dose of a single injection of kisspeptin-54 (50 nmol) and GnRH (50 nmol) on testicular degeneration.

Rats were randomized into five groups ($n = 5$ per group). At $t = 0$, rats received a single s.c. injection of either 0.9% saline, 0.5 nmol, 5 nmol or 50 nmol kisspeptin-54 or 50 nmol GnRH and were killed by decapitation 24 h following injection. The left testes ($n = 5$ per group) were immediately dissected and weighed. Following careful removal, the testes were prepared for histological analysis and assessed as described above ($n = 5$ per group).

(ii) Comparison of the effects of a single high-dose s.c. injection of kisspeptin-54 or GnRH on plasma LH, FSH and testosterone
In separate rats, we compared the effects of the same dose of a single s.c. injection of kisspeptin-54 (50 nmol) and GnRH (50 nmol) on plasma LH, FSH and testosterone at 60 min following injection. Rats were randomized into six groups ($n = 5$ per group). At $t = 0$, rats received a s.c. injection of either 100 μL 0.9% saline, 50 nmol kisspeptin-54 or 50 nmol GnRH. Rats were killed by decapitation at 60 min following injection. Trunk blood was collected from the rats killed at 60 min post injection, as described above, and stored at –20°C until measurement of LH, FSH and free testosterone.

Study D: Investigation of the effect of i.c.v. administration of kisspeptin-54 on testicular histology in adult male rats
i.c.v. cannulation and injections. Animals were implanted with permanent 22-gauge stainless steel i.c.v. cannulae projecting to the third cerebral ventricle (coordinates; 0.8 mm posterior to bregma on the midline and implanted 6.5 mm below the outer surface of the skull) as previously described (Rossi *et al.*, 1997). Following a 7 day recovery period, the animals were acclimatized to the injection procedure and to the guillotine apparatus to minimize the metabolic consequences of stress on the study day. Only animals with correct cannula placement, as confirmed by a sustained drinking response to i.c.v. angiotensin II (150 ng), were included in the studies. All studies were carried out in the early light phase (0800–1100 h). Kisspeptin-54 was dissolved in 0.9% saline and administered in a 5 μL volume via a stainless steel injector projecting 1 mm beyond the tip of the cannula. The injector was connected by polyethylene tubing (inner diameter, 0.5 mm; outer diameter, 1 mm) to a Hamilton syringe (Reno, NV, USA) in a syringe pump set to dispense 5 μL solution per minute.

i.c.v. study procedure. Rats were randomized into two groups ($n = 5$ per group) and i.c.v. injected with either 0.9% saline or 5 nmol kisspeptin-54. Rats were decapitated at 24 h following injection. The left testes ($n = 5$ per group) were immediately dissected and weighed, prepared for histological analysis and assessed as described above.

Study E: Effect of s.c. administration of kisspeptin-54 following pretreatment with a GnRH-R antagonist on testicular degeneration
(i) A study was first performed to confirm that cetrorelix, a GnRH-R antagonist, inhibits the kisspeptin-54-induced stimulation of the HPG axis.

In this study two groups of rats ($n = 5$ per group) were pretreated with s.c. injection of 0.9% saline (200 μL), and two separate groups of rats ($n = 5$ per group) were pretreated with s.c. injection of cetrorelix (200 nmol·200 μL⁻¹ saline). Thirty min later each group of rats were treated with either s.c. injection of 0.9% saline (200 μL) or kisspeptin-54 (50 nmol·200 μL⁻¹ saline) (Matsui *et al.*, 2004). The animals were decapitated, and their trunk blood was collected 60 min after kisspeptin-54 administration and stored as described above until measurement of LH, FSH and free testosterone.

(ii) Following confirmation that pretreatment with cetrorelix prevented s.c. kisspeptin-54 (50 nmol)-induced HPG axis stimulation, adult male rats were s.c. injected with kisspeptin-54 (50 nmol·200 μL⁻¹ saline) or 0.9% saline alone (200 μL) 30 min after pretreatment with either s.c. cetrorelix (200 nmol·200 μL⁻¹ saline) or 0.9% saline (200 μL). Rats were decapitated at 24 h following injection. The left testes ($n = 5$ per group) were immediately dissected and weighed, prepared for histological analysis and assessed as described above.

Study F: Testicular degeneration recovery study
We investigated whether the testicular degeneration following a single peripheral injection of high-dose kisspeptin-54 is reversible. Rats were randomized into three groups ($n = 5$ per group). At time zero ($t = 0$) on day 0 rats received a s.c. injection of either 0.9% saline, 50 nmol kisspeptin-54 or 50 nmol GnRH. Rats were killed by decapitation 2 months following injection. Trunk blood was collected and stored as described above until measurement of LH, FSH, free testosterone and inhibin B. The left testes ($n = 5$ per group) were immediately dissected, weighed and prepared for histological analysis and assessed as described above.

Hormone assays
Luteinising hormone and FSH levels in plasma were assayed by 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 (Beak *et al.*, 1998). The intra- and inter-assay coefficients of variation for the LH assay were 8.2% and 13.6% respectively, and 8.3% and 12.4% for the FSH assay. Free testosterone in plasma was measured by EIA (Diagnostic Systems Laboratories, Oxfordshire, UK). Inhibin B was measured by solid-phase sandwich ELISA (Oxford Bio-Innovation, Oxfordshire, UK) (Harris and Levine, 2003). The intra- and inter-assay coefficients of variation for the commercial assays were <10%.

Statistical analysis
Results are shown as mean values ± SEM. Plasma hormone measurements for all studies were compared by ANOVA with post hoc Tukey adjustment (Systat). The weights of the testes for all studies were compared by ANOVA with post hoc Tukey adjustment. In all cases $P < 0.05$ was considered to be statistically significant.

Materials
The drug and molecular target nomenclature used in this study conforms to the *BJP* Guide to Receptors and Channels

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(Alexander *et al.*, 2006). Kisspeptin-54 was synthesized by the Advanced Biotechnology Centre, Imperial College (London, UK). GnRH was purchased from Bachem (UK) Ltd. The GnRH-R antagonist cetrorelix was purchased from Serono Ltd (Middlesex, UK).

Results

Study A: Effect of continuous s.c. kisspeptin-54 administration (50 nmol·day^{-1}) for 1, 2 or 3 days on testicular histology and plasma inhibin B

(i) Testes: The absolute and relative weights of the left testes were not significantly changed following short-term 1, 2 or 3 day continuous s.c. kisspeptin-54 administration, compared with saline-treated controls. All saline-treated controls had normal testicular histology, with no seminiferous cell degeneration observed. However, administration of 50 nmol·day^{-1} kisspeptin-54 for 1, 2 or 3 days led to focal damage of the seminiferous tubules, which was present as early as 1 day post initiation of treatment. Individual tubules showed varying degrees of degeneration. There was variable cell maturation arrest, generation of multinucleated spermatid giant cells formed from round spermatids, sloughing and apoptosis of germ cells, with complete focal atrophy of germ cells (Figure 1A–D). There were no evident differences in Leydig cell morphology or cell number (Figure 1E,F). The median percentage [interquartile range] of seminiferous tubules showing degeneration following 1 day of continuous s.c. kisspeptin-54 administration was 41.2% [19%–44%]. The percentage of tubules showing degeneration following 2 or 3 days administration was similar to that following 1 day administration (29% [12.8%–42.7%] and 28.4% [12.8%–42.7%] respectively). (ii) Plasma inhibin B: Continuous s.c. administration of 50 nmol·day^{-1} kisspeptin-54 for 1 day had no effect on plasma inhibin B levels compared with saline-treated controls. However, following 2 days of s.c. kisspeptin-54 administration, plasma inhibin B levels were approximately 50% lower than the saline-treated controls. The plasma inhibin B levels remained significantly suppressed following 3 days kisspeptin-54 treatment (Figure 2).

Study B: (i) Testicular degeneration is present as early as 12 h following continuous s.c. kisspeptin-54 administration

The absolute and relative weights of the left testes were not significantly reduced following 6, 12 or 24 h continuous s.c. kisspeptin-54 administration, compared with saline-treated controls. All saline-treated controls had normal testicular histology, with no seminiferous tubule degeneration observed. Testicular degeneration was not observed following 6 h kisspeptin-54 administration, the earliest time point examined. Continuous s.c. administration of 50 nmol·day^{-1} kisspeptin-54 led to testicular degeneration following 12 h administration. In terms of histological features present, the degree of degeneration following 12 h of kisspeptin-54 administration was not as severe as that seen following administration for 24 h. Following 12 h of kisspeptin-54 administration there was moderate focal degeneration of the seminiferous tubules characterized by apoptosis and

sloughing of germ cells (Figure 1G,H). Following 24 h of administration, the degeneration was as severe and showed the same features as that observed following 24 h continuous administration in Study A. The median percentage [interquartile range] of seminiferous tubules showing degeneration following 12 h and 24 h of continuous s.c. kisspeptin-54 administration were 12 h: 18.1% [10.1%–26.1%]; 24 h: 38.9 [24.3%–47.9%].

(ii) Early hormonal profile

Continuous s.c. administration of 50 nmol·day^{-1} kisspeptin-54 for 6 h significantly increased plasma LH almost 15-fold compared with saline-treated controls. Plasma LH was significantly increased following 12 h of continuous s.c. kisspeptin-54 administration. However, although the kisspeptin-54-stimulated increase in plasma LH following 12 h of continuous s.c. administration was almost 10-fold greater than saline-treated controls, it was significantly lower than the kisspeptin-54-stimulated plasma LH level following 6 h administration. Following 24 h of continuous kisspeptin-54 administration plasma LH was increased twofold compared with saline-treated controls; however, this increase did not reach statistical significance and was significantly lower than the plasma LH levels reached following 6 h and 12 h of kisspeptin-54 administration (Figure 3A).

Continuous s.c. administration of 50 nmol·day^{-1} kisspeptin-54 for 6 h significantly increased plasma FSH approximately 3.5-fold compared with saline-treated controls. Plasma FSH remained significantly increased following 12 h of s.c. kisspeptin-54 administration, when it was approximately twofold greater than saline-treated controls. However, the kisspeptin-54-stimulated increase in plasma FSH following 12 h of s.c. administration was significantly lower than the kisspeptin-54-stimulated plasma FSH level following 6 h of administration. Following 24 h of kisspeptin-54 administration, plasma FSH was no longer significantly increased compared with saline-treated controls and was significantly lower that the plasma FSH levels reached following 6 h and 12 h of s.c. kisspeptin-54 administration (Figure 3B).

Continuous s.c. administration of 50 nmol·day^{-1} kisspeptin-54 for 6 h increased plasma free testosterone approximately threefold compared with saline-treated controls; however, this increase did not reach statistical significance ($P = 0.1$). Plasma free testosterone was significantly increased following 12 h of kisspeptin-54 administration compared with saline-treated controls and was approximately sevenfold greater than saline-treated controls. The absolute levels of plasma free testosterone following kisspeptin-54 administration for 12 h was similar to and not significantly different from the plasma free testosterone levels following 6 h administration (6 h: 31.4 ± 8.45 pg·mL^{-1}, 12 h: 34.4 ± 10.31 pg·mL^{-1}, $P = 1.0$). Following 24 h of kisspeptin-54 administration plasma free testosterone was no longer significantly increased compared with saline-treated controls (Figure 3C).

(iii) Kisspeptin-54 does not induce non-specific tissue degeneration

The Limulus Amoebocyte Lysate assay test (Associates of Cape Cod, Liverpool, UK) for pyrogen was negative, suggesting that
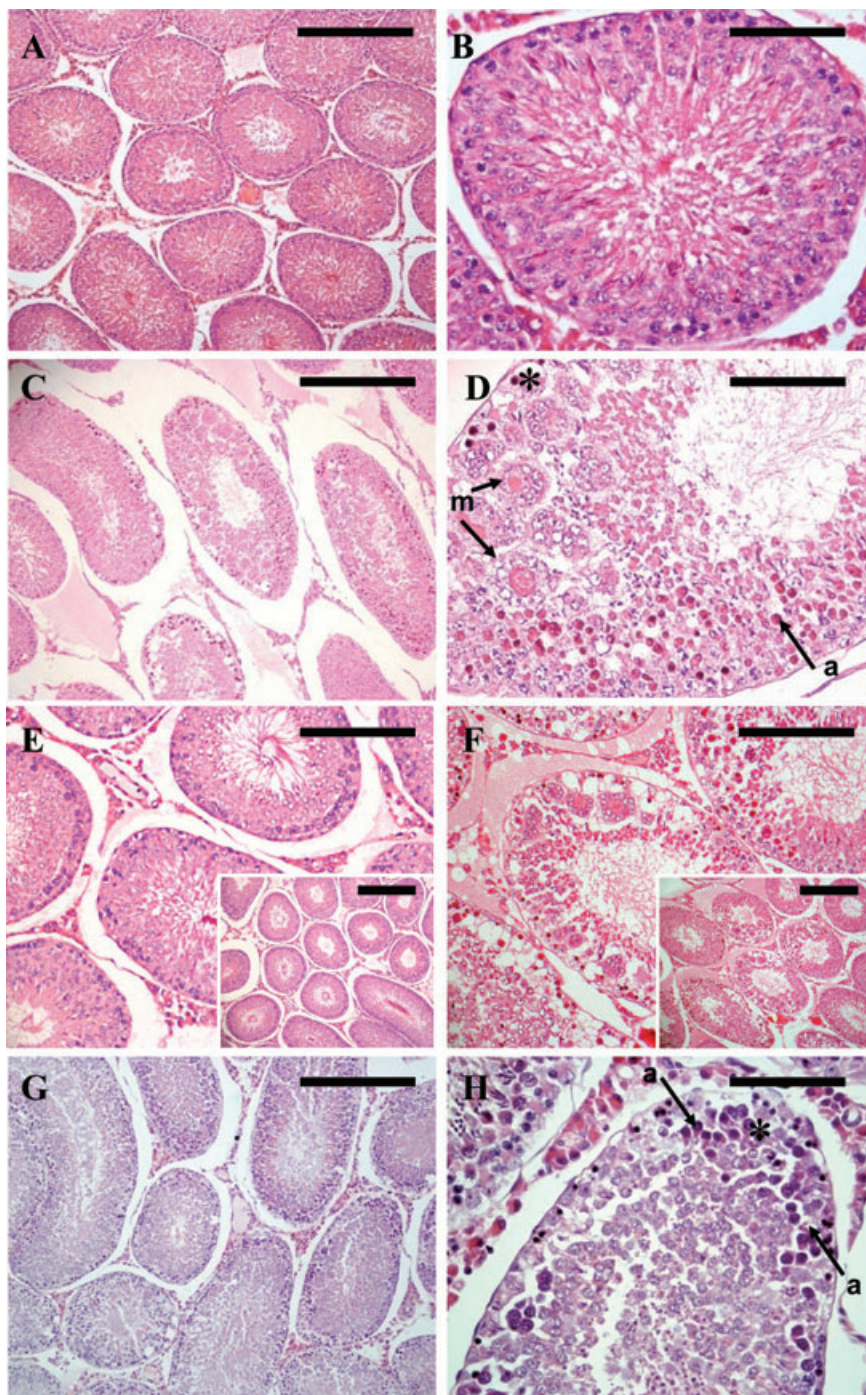
Figure 1 Testicular histology following continuous s.c. administration of kisspeptin-54 for 1 day (A–F) or 12 h (G & H). (A–F) Cross section of a seminiferous tubule assessed by light microscopy on H&E-stained sections from rats treated with continuous s.c. administration of (A, B & E) saline, or (C, D & F) 50 nmol·day^{-1} kisspeptin-54, for 1 day. (A) Representative section from a control animal showing tubules with normal maturation to late-stage elongated spermatids and a few spermatozoa. (B) Higher magnification of (A), to show a single tubule. (C) Representative section of an animal treated with continuous s.c. kisspeptin-54 administration for 1 day showing tubules with loss of seminiferous tubule structure with vacuolation, atrophy and formation of multinucleated giant cells. (D) Higher magnification of (C) to show a single tubule with severe necrotic degeneration of the germinal cells (asterisk), degeneration characterized by multinucleated giant cells (m, arrow) and apoptotic cells (a, arrow). (E & F) Comparison of histology between (E) a saline-treated animal and (F) a kisspeptin-54-treated animal showing comparable Leydig cell morphology (Original magnifications ×200. Scale bar represents 50 μm. Inset pictures, original magnifications ×100. Scale bar represents 100 μm). (G & H) Moderate testicular degeneration was present as early as following 12 h of continuous s.c. kisspeptin-54 administration. Cross section of a testicular tubule assessed by light microscopy on H&E-stained sections from adult rats treated with continuous s.c. administration of 50 nmol·day^{-1} kisspeptin-54, for 12 h. (G) Representative section of an animal treated with continuous s.c. kisspeptin-54 administration for 12 h showing seminiferous tubules with moderate degeneration. (H) Higher magnification of (G) showing a tubule with moderate degeneration of the germinal cells (asterisk) and apoptotic cells (a, arrow). (A, C & G: Original magnifications ×100. Scale bar represents 100 μm. B, D & H: Original magnifications ×400. Scale bar represents 25 μm). H&E, haematoxylin and eosin.

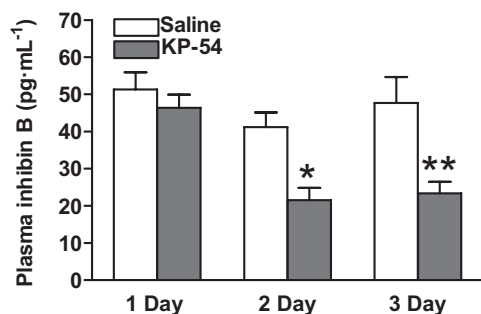Testicular effects of kisspeptin-54
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Figure 2 The effect of continuous, short-term (1, 2 and 3 day), s.c. administration of 50 nmol·day^{-1} kisspeptin-54 (KP-54) in adult male Wistar rats on plasma levels of inhibin B. All data are expressed as mean ± SEM. Significance is indicated by *$P < 0.05$, **$P < 0.01$ vs. saline-treated control ($n = 8$ per group).

the kisspeptin-54 used in these studies did not contain pyrogens. In addition, no other tissues histologically assessed [vas deferens, prostate, epididymis, liver, heart, lung, small intestine and spleen ($n = 5$ per group)] following continuous s.c. 50 nmol kisspeptin-54 administration for 24 h underwent degenerative changes and all were indistinguishable from saline-treated controls (data not shown).

(iv) A single s.c. injection of kisspeptin-54 leads to testicular degeneration

The absolute and relative weights of the left testes were not significantly reduced following a single s.c. injection of 50 nmol kisspeptin-54, compared with saline-treated controls, when assessed 24 h following injection. Interestingly, a single s.c. injection of 50 nmol kisspeptin-54 led to significant testicular degeneration compared with saline-treated controls, when observed 24 h after acute administration. All saline-treated animals had normal testicular histology, with no seminiferous tubule degeneration observed. The severity of degeneration seen 24 h after a single s.c. injection of 50 nmol kisspeptin-54 was comparable with that produced following continuous s.c. administration of 50 nmol of kisspeptin-54 over 24 h [Study A (i) and B (i) as described above]. The median percentage [interquartile range] of degenerated tubules following a single s.c. injection of 50 nmol kisspeptin-54 administration was similar to that produced by continuous s.c. administration of 50 nmol kisspeptin-54 over 24 h (continuous administration: 38.9% [24.3%–47.9%], single injection: 33.1% [28.8%–43.8%].

Study C: (i) Dose response of single s.c. kisspeptin-54 injections and comparison with a single s.c. GnRH injection on testicular degeneration

The absolute and relative weights of the left testes were not significantly reduced following a single s.c. injection of 0.5, 5 or 50 nmol kisspeptin-54, or 50 nmol GnRH compared with saline-treated control, when assessed 24 h following injection. All saline-treated animals had normal testicular histology, with no seminiferous cell degeneration observed (Figure 4A,B). A single s.c. injection of 0.5 nmol kisspeptin-54 did not lead to testicular degeneration at 24 h post injection.
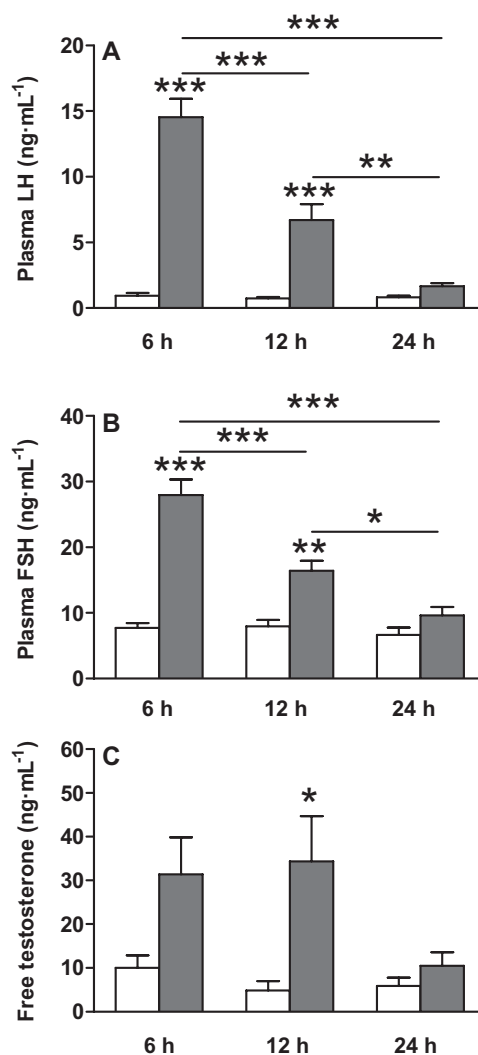

Figure 3 The effect of continuous, short-term (6, 12 and 24 h), s.c. administration of 50 nmol·day^{-1} kisspeptin-54 on the HPG axis. The effect of continuous, short-term (6, 12 and 24 h), s.c. administration of 50 nmol·day^{-1} kisspeptin-54 in adult male Wistar rats on plasma levels of (A) LH, (B) FSH and (C) free testosterone. All data are expressed as mean ± SEM. Significance is indicated by *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. saline-treated control, or a kisspeptin-54-treated group at a different time point ($n = 5$ per group). FSH, follicle-stimulating hormone; HPG, hypothalamic-pituitary gonadal; LH, luteinising hormone.

However, a s.c. injection of 5 nmol or 50 nmol kisspeptin-54 resulted in testicular degeneration. The median percentage [interquartile range] of seminiferous tubules showing signs of degeneration following 5 nmol s.c. kisspeptin-54 administration was 46% [12%–50.9%], and following 50 nmol kisspeptin-54 was 58.8% [40%–65.8%] (Figure 4C,D). The features were comparable to those described above [Study B (iv)].

A single s.c. injection of 50 nmol GnRH also caused degeneration of the testes at 24 h post injection. Three animals showed severe testicular degeneration following acute GnRH administration, which was comparable, in terms of the histological features seen, with that induced by a single s.c. injection of 5 and 50 nmol kisspeptin-54 (Figure 4E,F). However, two animals did not experience testicular degeneration in any
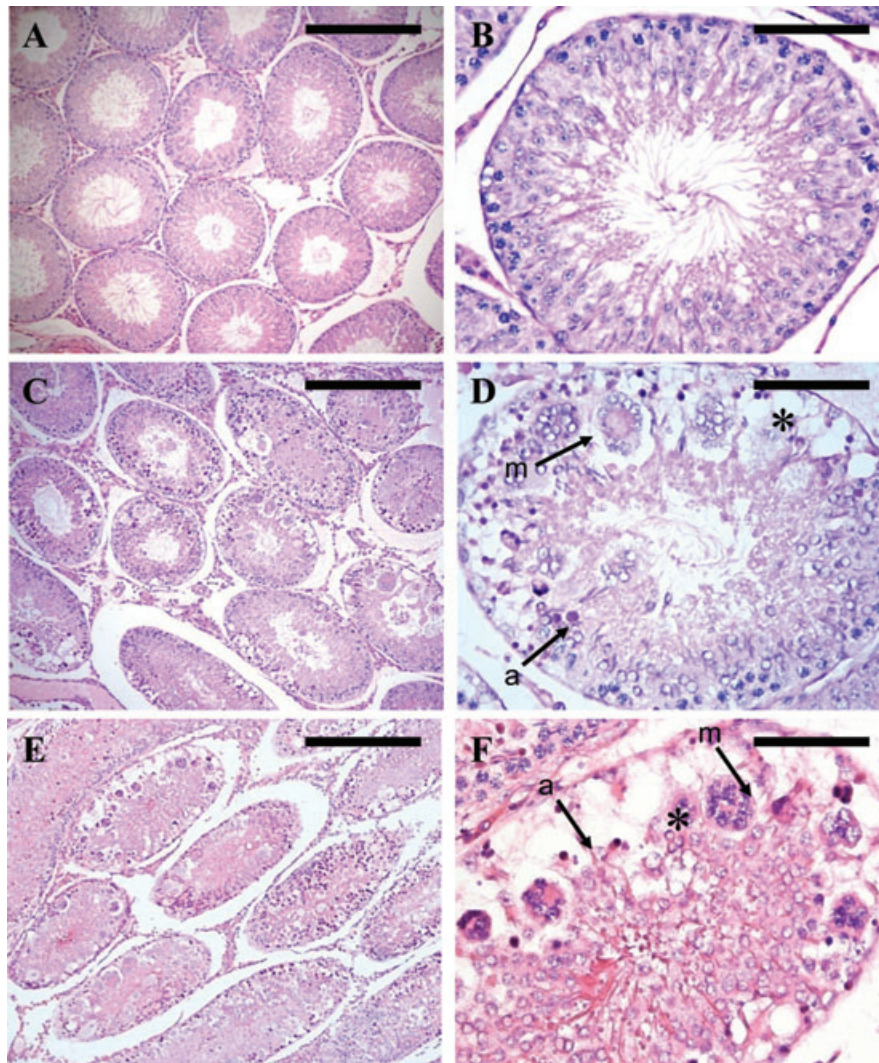
Figure 4 Testicular histology following a single s.c. injection of 50 nmol kisspeptin-54 or GnRH in the adult rat. Testicular histology was assessed 24 h following injection. Cross sections of testicular tubules assessed by light microscopy on H&E-stained sections from rats treated with single s.c. injection of (A & B) saline, (C & D) kisspeptin-54 (50 nmol) or (E & F) GnRH (50 nmol). (A) Representative section from a control animal showing tubules with normal maturation to late-stage elongated spermatids and a few spermatozoa. (B) Higher magnification of (A) to show a single tubule. (C) Representative section of an animal treated with a single s.c. injection of 50 nmol kisspeptin-54 showing severe testicular degeneration. (D) Higher magnification of (C) to show a tubule with severe necrotic degeneration and atrophy of the germinal cells (asterisk), degeneration characterized by multinucleated giant cells (m, arrow) and apoptotic cells (a, arrow). (E & F) Representative section of an animal treated with a single s.c. injection of 50 nmol GnRH showing the same features as described for kisspeptin-54 in C & D. (A, C & E: Original magnifications ×100. Scale bar represents 100 μm. B, D & F: Original magnifications ×400. Scale bar represents 25 μm). GnRH, gonadotrophin-releasing hormone; H&E, haematoxylin and eosin.

of the tubules assessed, reducing the median percentage of tubules showing degeneration. The percentage of tubules showing degeneration for the five animals injected with 50 nmol GnRH was 0%, 0%, 19.6%, 30% and 42.2%. The median percentage [interquartile range] of seminiferous tubules showing signs of degeneration following GnRH administration was 19.6% [0%–30%].

(ii) Circulating hormones following equal s.c. doses of kisspeptin-54 and GnRH
A single s.c. injection of 50 nmol kisspeptin-54 or 50 nmol GnRH significantly increased plasma LH at 60 min post injection approximately fivefold and sixfold respectively,

compared with saline-treated control. The plasma LH levels following kisspeptin-54 or GnRH administration were not significantly different from each other (Figure 5A).

A single s.c. injection of 50 nmol kisspeptin-54 or 50 nmol GnRH significantly increased plasma FSH at 60 min post injection approximately twofold and threefold respectively, compared with saline-treated control. A s.c. injection of 50 nmol GnRH produced a significantly greater increase in plasma FSH than 50 nmol kisspeptin-54 at 60 min post injection (Figure 5B).

A single s.c. injection of 50 nmol kisspeptin-54 or 50 nmol GnRH significantly increased plasma free testosterone at 60 min post injection by approximately 2.5-fold and threefold respectively, compared with saline-treated control. The plasma
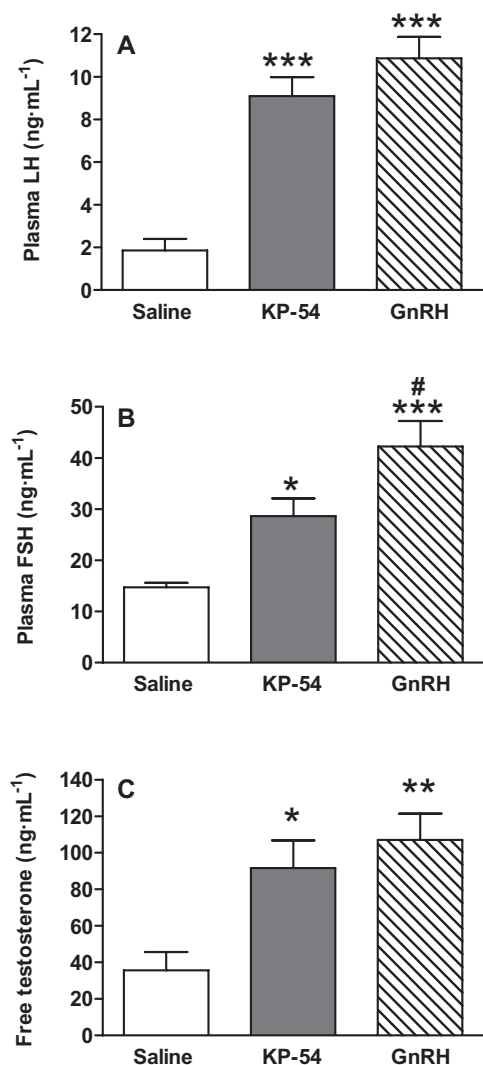
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Figure 5 Comparison of s.c. kisspeptin-54 and GnRH on hormones of the HPG axis. Effect of s.c. administration of 50 nmol kisspeptin-54 (KP-54) and 50 nmol GnRH on plasma levels of (A) LH, (B) FSH and (C) free testosterone in adult male Wistar rats at 60 min post injection. All data are expressed as mean ± SEM. Significance is indicated by *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. saline-treated control, or #$P < 0.05$, GnRH vs. kisspeptin-54 ($n = 5$ per group). FSH, follicle-stimulating hormone; GnRH, gonadotrophin-releasing hormone; HPG, hypothalamic-pituitary gonadal; LH, luteinising hormone.

free testosterone levels following kisspeptin-54 or GnRH were not significantly different from each other (Figure 5C).

The increases in plasma gonadotrophins induced by 50 nmol kisspeptin-54 or GnRH were diminished by 24 h following injection. At this time point there were no significant differences in plasma levels of these hormones between treatment groups (LH: saline 0.68 ± 0.25 ng·mL^{-1}, kisspeptin-54 0.42 ± 0.10 ng·mL^{-1}, GnRH 0.58 ± 0.26 ng·mL^{-1}, no significance. FSH: saline 11.34 ± 0.74 ng·mL^{-1}, kisspeptin-54 15.29 ± 1.24 ng·mL^{-1}, GnRH 14.32 ± 2.06 ng·mL^{-1}, no significance).

Study D: i.c.v. kisspeptin-54 on testicular degeneration
The absolute and relative weights of the left testes were not significantly reduced following i.c.v. administration of 5 nmol

kisspeptin-54, compared with saline-treated controls, when assessed 24 h following injection. No saline-treated animals had abnormal testicular histology (Figure 6A,B). A single i.c.v. injection of 5 nmol kisspeptin-54 led to testicular degeneration in all animals, when the testes were assessed 24 h following i.c.v. injection. However, there was considerable variation in the number of tubules showing degeneration between animals. The histological features observed in the degenerated tubules were similar to those described above [Study A (i), B (i) and B (iv)] (Figure 6C,D). The median percentage [interquartile range] of degenerated tubules in the group that received 5 nmol kisspeptin-54 was 13.3% [4.7%–25%].

Study E: Pretreatment with a GnRH-R antagonist on kisspeptin-54-induced testicular degeneration

(i) Subcutaneous pretreatment with cetrorelix (200 nmol) abolished the s.c. induced stimulation of the HPG axis by 50 nmol kisspeptin-54. A s.c. injection of 50 nmol kisspeptin-54 dramatically increased plasma LH ($P < 0.001$), FSH ($P < 0.001$) and free testosterone ($P < 0.001$) compared with saline-treated controls. Gonadotrophin and testosterone responses to 50 nmol kisspeptin-54 were blocked in rats pretreated with 200 nmol of GnRH-R antagonist cetrorelix ($P < 0.001$ cetrorelix plus kisspeptin-54 vs. kisspeptin-54 alone). There were no significant differences between the gonadotrophin or testosterone levels following treatment with cetrorelix alone, cetrorelix plus kisspeptin-54 or saline (Figure 7).

(ii) A single s.c. injection of 50 nmol kisspeptin-54 alone produced testicular degeneration in all animals at 24 h following administration. Histological features were consistent with those described above [Study B (iv) and Study C (i)], and, as previously seen following kisspeptin-54 administration, the number of seminiferous tubules showing degeneration varied between the animals. The percentage of tubules showing degenerative changes per testis section scored ranged from 2.7% to 50%. Pretreatment with the GnRH-R antagonist cetrorelix (200 nmol) completely prevented the kisspeptin-54-induced testicular degeneration in all animals, with none of the seminiferous tubules examined in this group showing signs of damage. No saline-treated rats or rats treated with cetrorelix alone showed any testicular degeneration (Figure 8).

Study F: Testicular degeneration induced by high-dose s.c. single injection of kisspeptin-54 and GnRH persists 2 months after injection
(i) Testicular histology. A single s.c. injection of 50 nmol kisspeptin-54 led to a significant decrease in testis weight at 2 months following injection compared with saline-treated control. A single s.c. injection of 50 nmol GnRH caused a trend towards decreased testis weight at 2 months following injection, but this effect did not achieve statistical significance (left testis weight, saline 1.98 ± 0.08 g; kisspeptin-54 1.51 ± 0.13 g, $P < 0.05$ vs. saline; GnRH 1.70 ± 0.10 g,
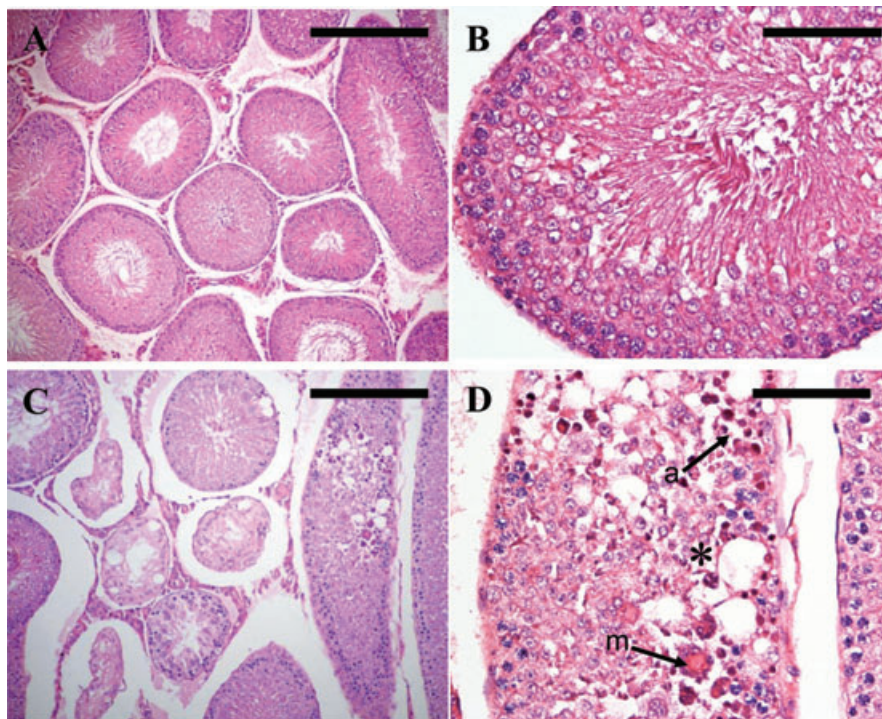
Figure 6 Testicular histology following a single i.c.v. injection of 5 nmol kisspeptin-54 in the adult rat. Testicular histology was assessed 24 h following injection. Cross sections of testicular tubules assessed by light microscopy on H&E-stained sections from rats treated with a single i.c.v. injection of (A & B) saline, or (C & D) 5 nmol kisspeptin-54. (A) Representative section from a control animal showing normal maturation to late-stage elongated spermatids and a few spermatozoa. (B) Higher magnification of (A) to show a single tubule. (C) Representative section from an animal that received 5 nmol i.c.v. kisspeptin-54, showing a severe testicular degeneration. (D) Higher magnification of (C) to show a tubule with severe necrotic degeneration and atrophy of the germinal cells (asterisk), degeneration characterized by multinucleated giant cells (m, arrow) and apoptotic cells (a, arrow). (A & C: Original magnifications ×100. Scale bar represents 100 μm. B & D: Original magnifications ×400. Scale bar represents 25 μm). H&E, haematoxylin and eosin; i.c.v., intracerebroventricular.

$P = 0.06$ vs. saline). There was no significant difference in testis weight between kisspeptin-54- and GnRH-treated rats.

In addition, the testicular degeneration produced by a single s.c. injection of 50 nmol kisspeptin-54 or 50 nmol GnRH was still present at 2 months following injection. The median percentage [interquartile range] of seminiferous tubules showing degeneration at 2 months following a single s.c. injection of high-dose kisspeptin-54 was 31% [13%–31%]. Kisspeptin-54 caused testicular degeneration in all animals treated. The median percentage [interquartile range] of seminiferous tubules showing degeneration at 2 months following a single s.c. injection of high-dose GnRH was 20% [0%–21%]. Two animals treated with GnRH did not experience testicular degeneration in any of the tubules assessed, reducing the median percentage of tubules showing degeneration. The percentage of tubules showing degeneration for the five animals injected with 50 nmol GnRH was 0%, 0%, 20%, 25.7% and 21.1%. The histological changes observed in the kisspeptin-54- and GnRH-treated animals were comparable. Histopathology revealed loss of seminiferous tubular structure, with vacuolation, atrophy and tubular hyalinization as common features (Figure 9). There were no evident differences in Leydig cell morphology or number (Figure 10).

(ii) Circulating hormones at 2 months following a single s.c. injection of kisspeptin-54 or GnRH. A single s.c. injection of 50 nmol kisspeptin-54 or 50 nmol GnRH had no effect on

circulating levels of LH or FSH at 2 months following injection (Figure 11A,B). The single s.c. injection of 50 nmol kisspeptin-54 or 50 nmol GnRH led to a trend towards decreased free testosterone at 2 months following injection, but this did not reach statistical significance (Figure 11C). The single s.c. injection of 50 nmol kisspeptin-54 led to a significant 2.5-fold decrease in plasma inhibin B ($P < 0.05$). There was also a trend towards decreased plasma inhibin B following a single s.c. injection of 50 nmol GnRH, but this effect did not reach statistical significance ($P = 0.06$) (Figure 11D).

Discussion

Our data suggest that acute administration of kisspeptin-54 can cause testicular degeneration via a GnRH-mediated effect. These results have important implications for targeting the kisspeptin system to treat reproductive disease.

We have previously shown that stimulation of the HPG axis following continuous s.c. administration of 50 nmol·day^{-1} kisspeptin-54 is completely abolished by 2 days of continuous treatment (Thompson *et al.*, 2006). In the present study continuous s.c. administration of 50 nmol·day^{-1} kisspeptin-54 led to a dramatic increase in plasma LH, FSH and testosterone at 6 h, and while still largely increased at 12 h of continuous treatment there was a significant decrease in plasma gonadotrophins over time. These results suggest that, although
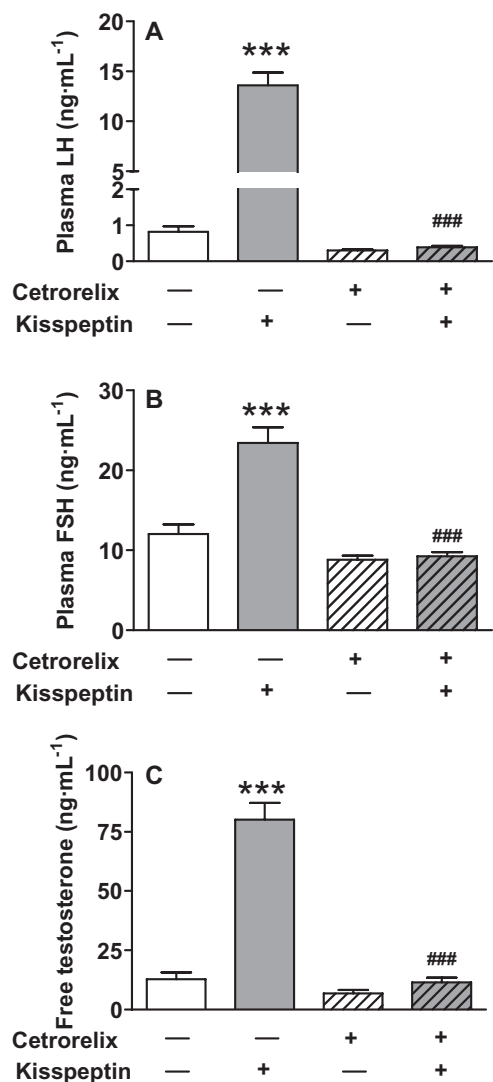
Figure 7 Plasma gonadotrophins and testosterone following a single s.c. injection of kisspeptin-54 following pretreatment with a GnRH-R antagonist in the adult male rat. The effects of s.c. administration of 50 nmol kisspeptin-54 30 min following s.c. pretreatment with a GnRH antagonist, cetrorelix (200 nmol) or saline alone in adult male Wistar rats on plasma levels of (A) LH, (B) FSH and (C) free testosterone at 60 min post injection of kisspeptin-54. All data are expressed as mean ± SEM. Significance is indicated by ***$P < 0.001$ vs. saline-treated control, or ###$P < 0.001$, cetrorelix plus kisspeptin-54 vs. kisspeptin-54 alone. There were no significant differences between cetrorelix- or cetrorelix plus kisspeptin-54-treated animals and animals treated with saline only ($n = 8$ per group). GnRH, gonadotrophin-releasing hormone; GnRH-R, GnRH-receptor; FSH, follicle-stimulating hormone; LH, luteinising hormone.

stimulation of the HPG axis was still evident over a 24 h period, it was starting to become desensitized to the stimulatory effects of kisspeptin-54. However, the results of the current study suggest that kisspeptin-induced testicular degeneration in adult male rats is not due to the desensitization of the HPG axis to the effects of kisspeptin-54. In the present study the continuous s.c. administration of kisspeptin-54 led to testicular degeneration after only 12 h of administration. This effect was thus apparent before the HPG axis was completely desensitized to the stimulatory effects of

kisspeptin-54. In addition, a single s.c. injection of high-dose kisspeptin-54 (50 nmol) also led to severe testicular degeneration. Therefore, the testicular degeneration seen following kisspeptin-54 administration is unlikely to be explained by the desensitization or down-regulation of the HPG axis. Importantly, s.c. kisspeptin-54 administration did not lead to degenerative changes in the vas deferens, prostate, epididymis, liver, heart, lung, small intestine or spleen. GPR54 mRNA has been detected in moderate levels in the rat small intestine (Lee *et al*., 1999; Terao *et al*., 2004) and liver (Lee *et al*., 1999), but has not been detected in the heart, lung or spleen (Lee *et al*., 1999; Terao *et al*., 2004). GPR54 expression has not been reported in the vas deferens, prostate and epididymis. The lack of kisspeptin-54-induced morphological changes in tissues that do or do not express GPR54 suggests that the degeneration caused by kisspeptin-54 administration in the testes is a specific effect.

The testicular degeneration following a single s.c. injection of kisspeptin-54 was dose-dependent. A s.c. injection of 0.5 nmol kisspeptin-54 has previously been shown to produce a submaximal increase in plasma LH (Thompson *et al*., 2006), but in the present study had no effect on the testicular histology. However, administration of 5 or 50 nmol kisspeptin-54, which produce a maximal increase in plasma LH (Thompson *et al*., 2006), did cause testicular degeneration. We hypothesized that the testicular degenerative effects of continuous or acute peripheral kisspeptin-54 administration may be a result of hyper-stimulation of the HPG axis. If this is the case, the effects are likely to be centrally mediated via an increase in hypothalamic GnRH release. Interestingly, in the present study we have shown that a single s.c. injection of 50 nmol GnRH can also cause testicular degeneration. Although not all animals were affected, three of the five GnRH-treated animals displayed the same histological features and severity of degenerative effects as those following a single s.c. injection of 50 nmol kisspeptin-54. To the best of our knowledge there are no reported studies on the acute effects of GnRH on the testes. However, in accord with our findings, the single injection of a potent GnRH super-agonist has previously been shown to cause severe damage to the rat testis within 24 h of administration (Habenicht *et al*., 1985). In the present study, the increase in plasma LH and testosterone were also comparable following a single s.c. injection of either 50 nmol kisspeptin-54 or 50 nmol GnRH at 60 min following administration. Therefore, at this dose and time point, kisspeptin-54 and GnRH are equipotent in stimulating LH and testosterone release and both cause comparable testicular degeneration. The stimulation of the HPG axis following a single injection of 50 nmol kisspeptin-54 or GnRH was lost by 24 h following injection. A single s.c. injection of a similar dose of kisspeptin-54 to that used in the present study (100 nmol·kg^{-1}) to adult male rats has been shown to maximally stimulate plasma LH and FSH at 2 h after injection. However, by 4 h following injection, plasma LH was not significantly different from saline-treated controls (Matsui *et al*., 2004). The kisspeptin-54-induced hyper-stimulation of the HPG axis is therefore likely to be lost within 4 h. To further support the hypothesis that kisspeptin-54-induced testicular degeneration is gonadotrophin-mediated, we have demonstrated that a single i.c.v. injection of high-dose kisspeptin-54
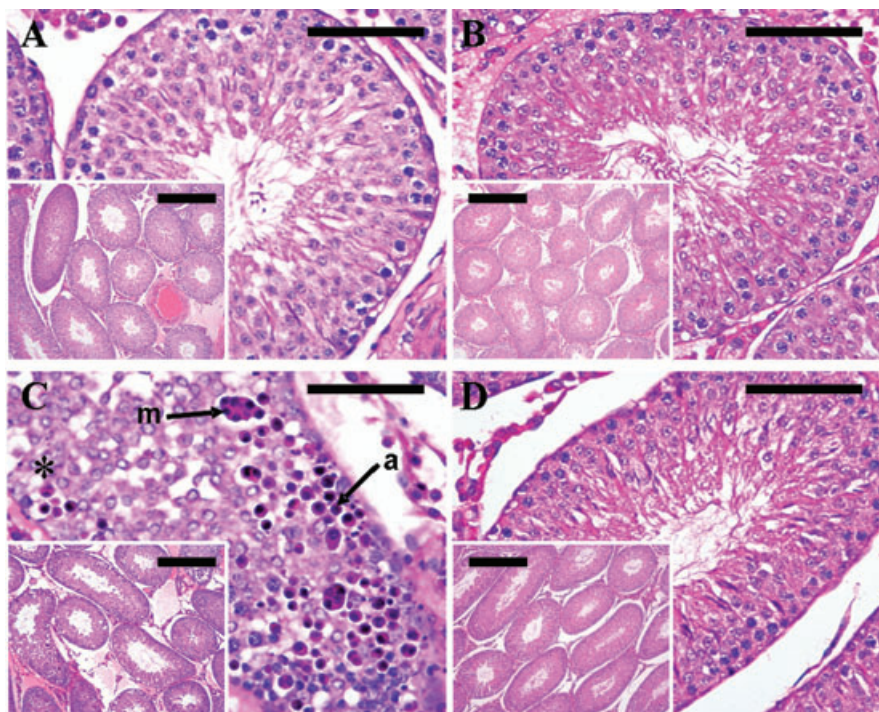
Figure 8 Testicular histology following a single s.c. injection of kisspeptin-54 following pretreatment with a GnRH-R antagonist in the adult male rat. The effect of s.c. administration of 50 nmol kisspeptin-54 30 min following s.c. pretreatment with a GnRH antagonist, cetrorelix (200 nmol) or saline alone in adult male Wistar rats on testicular histology. Testicular histology was assessed 24 h following injection of kisspeptin-54. Cross sections of testicular tubules assessed by light microscopy on H&E-stained sections. (A) Representative section from a control animal showing normal maturation to late-stage elongated spermatids and a few spermatozoa. (B) Representative section from an animal treated with cetrorelix alone showing normal maturation to late-stage elongated spermatids and a few spermatozoa. (C) Representative sections of an animal treated with s.c. kisspeptin-54 showing a tubule with severe necrotic degeneration and atrophy of the germinal cells (asterisk), degeneration characterized by multinucleated giant cells (m, arrow) and apoptotic cells (a, arrow). (D) Representative section from an animal pretreated with cetrorelix before a s.c. injection of kisspeptin-54, showing normal maturation to late-stage elongated spermatids and a few spermatozoa. (Original magnifications ×400. Scale bar represents 25 μm. Inset pictures, original magnifications ×100. Scale bar represents 100 μm). GnRH, gonadotrophin-releasing hormone; GnRH-R, GnRH-receptor; H&E, haematoxylin and eosin.

(5 nmol) led to testicular degeneration. This dose of kisspeptin-54 is higher than the dose required for maximal stimulation of gonadotrophin and testosterone release (Thompson *et al.*, 2004; Navarro *et al.*, 2005a,b; Patterson *et al.*, 2006). Although the percentage of damaged seminiferous tubules varied between animals, there was qualitative evidence of tubule degeneration following central administration of high-dose kisspeptin-54, in marked contrast to saline-treated animals that showed no degeneration. It therefore appears that the effects of kisspeptin-54 on testicular degeneration are likely to be centrally mediated, rather than a direct effect of kisspeptin-54 on the testes. Although there are no reported studies on the effects of acute LH administration on testicular histology, treating adult hypophysectomized male rats with LH twice daily for 2 weeks also dose-dependently disrupts the seminiferous tubules, with comparable histological features (Kerr and Sharpe, 1986). Interestingly, the single or repeated peripheral injection of pharmacological doses of human chorionic gonadotrophin (hCG), which acts via the LH-receptor, can lead to testicular degeneration in rats, with patchy necrosis and tubular atrophy (Leon *et al.*, 1987; Kerr and Sharpe, 1989; Chatani, 2006; Karaman *et al.*, 2006). Although hCG is used clinically to stimulate testicular descent and stimulate spermatogonia maturation in cryptorchid boys (Lala *et al.*, 1997), similar testicular degenerative changes have

been observed in follow-up of hCG-treated boys (Hjertkvist *et al.*, 1993; Heiskanen *et al.*, 1996). The time course of effects and histological features that are manifested following a single injection of hCG, are similar to those seen in the present study following peripheral kisspeptin-54 administration, leading to degeneration of germ cells in focal areas between 6 and 12 h following injection (Leon *et al.*, 1987; Kerr and Sharpe, 1989). In the present study the high-dose s.c. kisspeptin-54-induced testicular degeneration was completely prevented by pretreatment with a GnRH-R antagonist, strongly supporting the hypothesis that the testicular degeneration seen in the present study following high doses of kisspeptin-54 is a result of hyper-stimulation of the HPG axis and is driven by the marked, and presumably supraphysiological, increase in plasma LH.

It is possible that the degeneration represents in part a direct testicular effect of kisspeptin-54. GPR54 has been reported to be expressed in the testis, although the cell type and physiological significance has not been determined (Kotani *et al.*, 2001; Ohtaki *et al.*, 2001; Funes *et al.*, 2003; Terao *et al.*, 2004). One possibility is that the testicular degeneration seen following kisspeptin administration may represent changes in testicular blood flow. Decreases in testicular blood flow result in focal damage of the seminiferous tubules (Markey *et al.*, 1994; Bergh *et al.*, 2001). GPR54 mRNA is
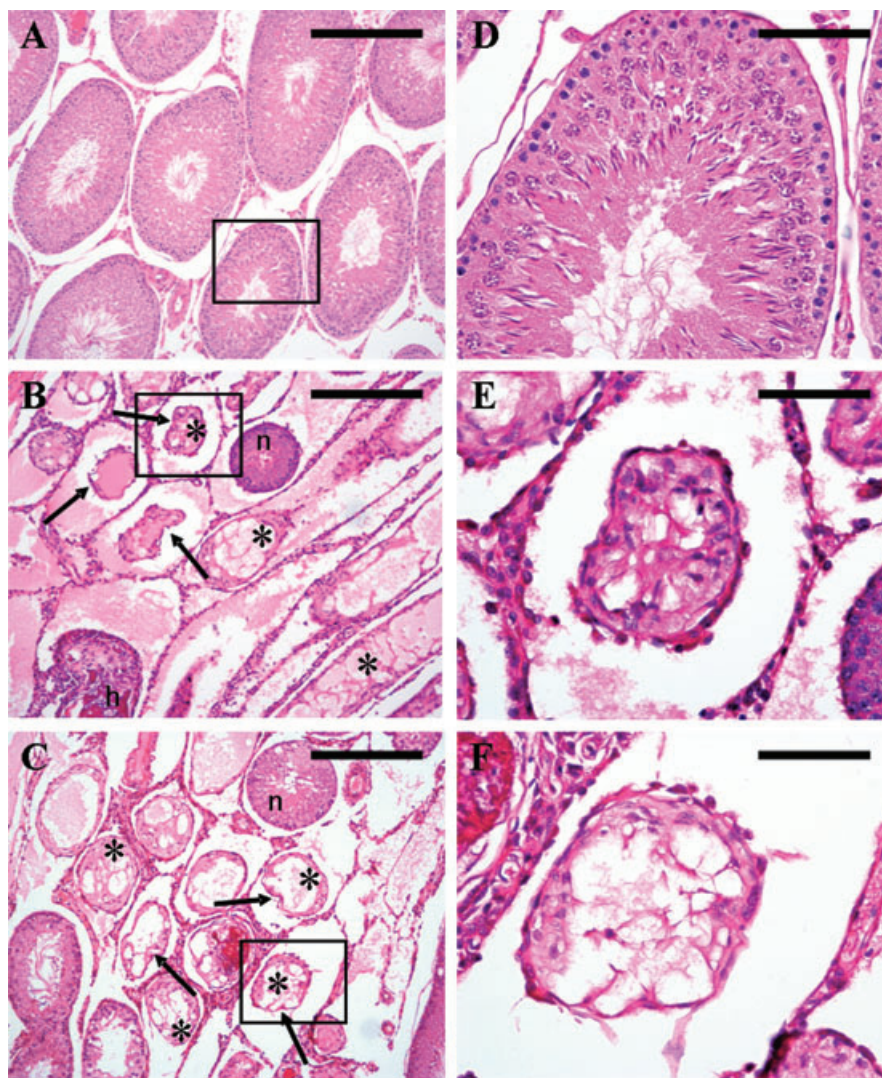
Figure 9 Seminiferous tubule histology at 2 months following a single s.c. injection of kisspeptin-54 (50 nmol) or GnRH (50 nmol) in the adult male rat. Cross sections of testicular tubules assessed by light microscopy on H&E-stained sections. (A) Representative section from a control animal showing tubules with normal maturation to late-stage elongated spermatids and a few spermatozoa. (B) Representative section of an animal treated with a single s.c. injection of 50 nmol kisspeptin-54 showing tubules with loss of seminiferous tubular structure (arrows) with vacuolation and atrophy (asterisk) and tubular hyalinization (h) as common features. The section also shows an apparently normal seminiferous tubule alongside the degenerated tubules (n). (C) Representative section of an animal treated with a single s.c. injection of 50 nmol GnRH showing the same features as described for kisspeptin-54 in (B). (Original magnifications ×100. Scale bar represents 100 μm). (D–F) Higher magnification of (A–C), to show a single tubule (boxed section) from each treatment group. (Original magnifications ×400. Scale bar represents 25 μm). GnRH, gonadotrophin-releasing hormone; H&E, haematoxylin and eosin.

expressed in the smooth muscle of the aorta, coronary artery and umbilical vein (Mead *et al.*, 2007b), but its expression in the blood vessels of the testis has not been investigated. Kisspeptins have been shown to act as constrictors of isolated rings of coronary artery *in vitro* (Mead *et al.*, 2007b). One study has detected kisspeptin mRNA in the human testis by quantitative reverse transcriptase polymerase chain reaction (Ohtaki *et al.*, 2001), but it has not been detected in the testis of the rat (Terao *et al.*, 2004). Thus it is possible that kisspeptins may play an autocrine or paracrine role in the testis. However, our finding that pretreatment with a GnRH-R antagonist prevented the testicular degeneration caused by kisspeptin-54 suggests that it is unlikely that a direct effect of kisspeptin-54 on the testes plays any major role in inducing testicular degeneration.

The testicular degeneration following continuous kisspeptin-54 administration was accompanied by a significant decrease in circulating inhibin B after 2 days of continuous administration. Plasma inhibin B, produced predominantly by the Sertoli cells, usually correlates with Sertoli cell number (Ramaswamy *et al.*, 1999; Sharpe *et al.*, 1999). Inhibin B has been proposed as a sensitive endocrine marker reflecting the state of spermatogenesis, with low levels reflecting a disturbance of spermatogenesis (Pierik *et al.*, 2003). Interestingly, the kisspeptin-54- and GnRH-induced testicular degeneration following a single 50 nmol s.c. injection remained visible 2 months after administration. This degeneration was associated with a trend towards decreased plasma testosterone. There was no obvious change in the morphology or cell number of the Leydig cells between treatment
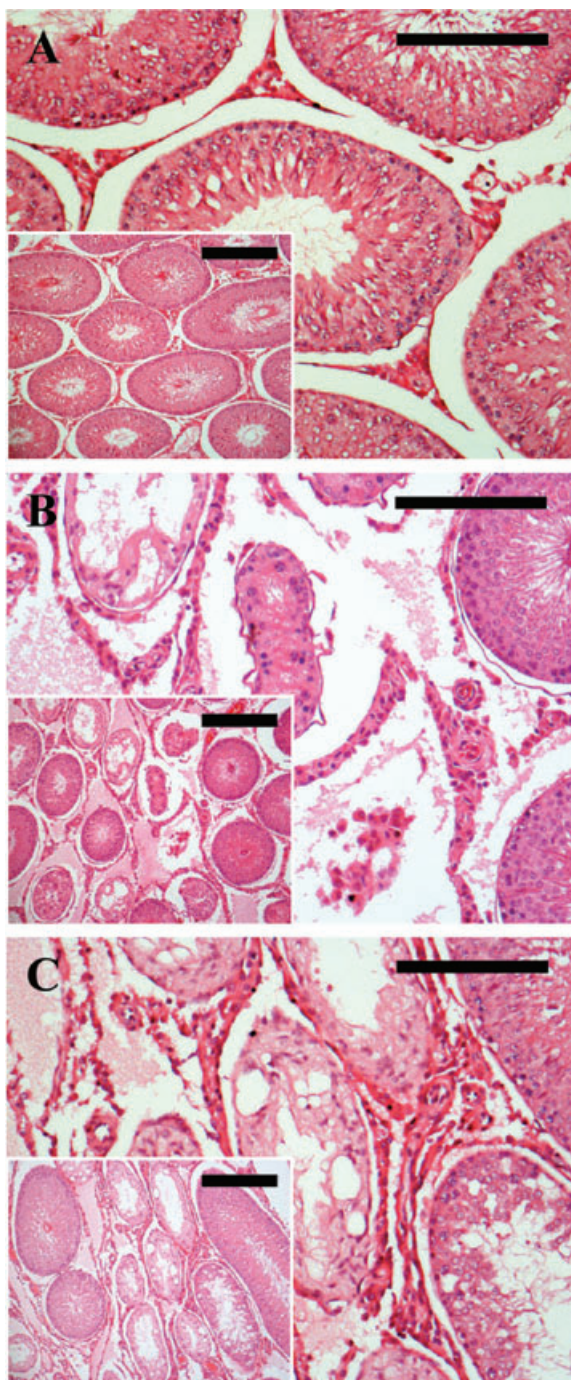
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Figure 10 Leydig cell histology at 2 months following a single s.c. injection of kisspeptin-54 (50 nmol) or GnRH (50 nmol) in the adult male rat. Cross sections of testicular tubules assessed by light microscopy on H&E-stained sections. (A) Representative section from a control animal showing normal seminiferous tubules and Leydig cell morphology. (B) Representative section of an animal treated with a single s.c. injection of 50 nmol kisspeptin-54 showing normal Leydig cell morphology and cell number surrounding degenerated seminiferous tubules. (C) Representative section of an animal treated with a single s.c. injection of 50 nmol GnRH showing the same features as described for kisspeptin-54 in (B). (Original magnifications × 200. Scale bar represents 50 μm. Inset pictures, original magnifications × 100. Scale bar represents 100 μm). GnRH, gonadotrophin-releasing hormone; H&E, haematoxylin and eosin.

groups. However, it is possible that the trend towards a decrease in testosterone may indicate a defect in the steroidogenic potential of the Leydig cells. It would be interesting to investigate this possibility further. There was a significant decrease in plasma inhibin B at 2 months following injection suggesting that the visible damage may reflect a persistent disruption of spermatogenesis (Pierik *et al.*, 2003). It would be useful to investigate whether fertility is disturbed and whether the testes may recover from the degenerative effects at a later time point after cessation of treatment. A single oral dose of the indenopyridine, CDB-4022, which is in development as a male contraceptive, leads to testicular degeneration and a progressive decrease in testes weight and plasma inhibin B levels that reach non-detectable levels by 14 days (Koduri *et al.*, 2008). The long-term suppression of plasma inhibin B may be attributable to a lasting effect on the Sertoli cell and/or the lack of differentiating germ cells in the seminiferous tubules (Koduri *et al.*, 2008). Sustained testicular degeneration has also been observed in the adult rat following a single injection of hCG at 3 months following injection (Leon *et al.*, 1987). Leon *et al.* suggest that the extreme germ cell depletion, tubular hyalinization and loss of cellular content were likely to represent the late evolution of the acute damage. The mechanism by which an acute hyper-stimulation of the HPG axis is responsible for sustained testicular degeneration is unclear, but may result from the acutely raised plasma LH. The mechanism by which hCG causes testicular damage appears to involve induction of local testicular production of inflammatory mediators and vascular regulators, resulting in testis inflammation that impairs circulation and results in hypoxia (Chatani, 2006; Bergh and Soder, 2007).

Thus in summary, pharmacological doses of kisspeptin-54 can cause testicular degeneration in the adult male rat, which is likely to be a result of central hyper-stimulation of the HPG axis. Importantly, the effects of acute kisspeptin-54 on the testes were dose-dependent. It is possible to stimulate the HPG axis with kisspeptin-54 without causing damage to the testes. The kisspeptins therefore represent a potential tool for HPG axis manipulation in humans, but our results suggest that the doses used must be chosen with considerable care.
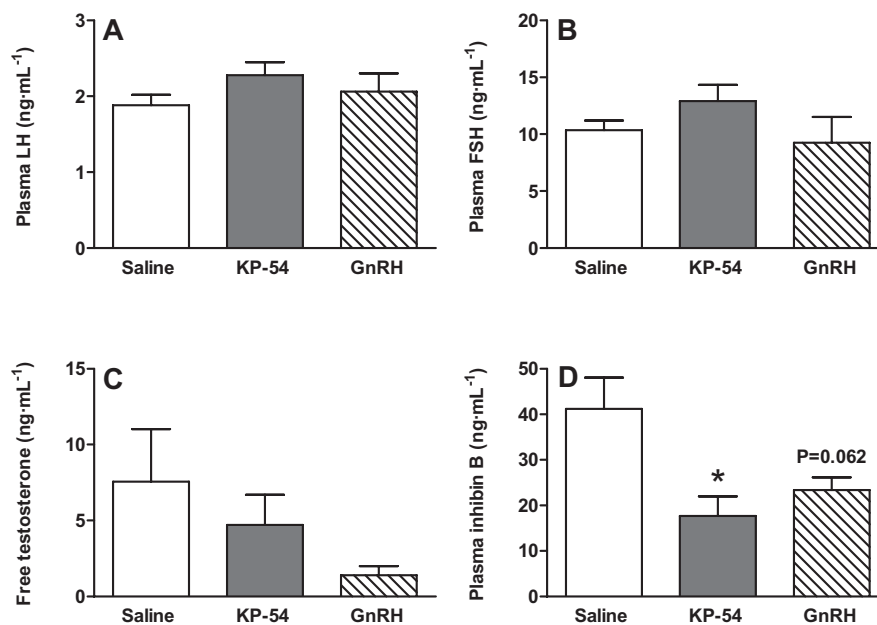
Acknowledgements

Figure 11 Plasma hormone levels at 2 months following a single injection of kisspeptin-54 or GnRH. The effects of a single s.c. injection of 50 nmol kisspeptin-54 (KP-54) or GnRH in adult male Wistar rats on plasma levels of (A) LH, (B) FSH, (C) free testosterone and (D) inhibin B at 2 months following injection. All data are expressed as mean ± SEM. Significance is indicated by *$P < 0.05$ vs. saline-treated control ($n = 5$ per group). FSH, follicle-stimulating hormone; GnRH, gonadotrophin-releasing hormone; LH, luteinising hormone.

Conflict of interest

None.

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