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Title: 'Central and peripheral administration of kisspeptin-10 stimulates the hypothalamic-pituitary-gonadal axis'

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Running Title: Kisspeptin stimulates the HPG axis

**Abstract**

Kisspeptin is the peptide product of the KiSS-1 gene and the endogenous agonist for the GPR54 receptor. Recent evidence suggests the kisspeptin/GPR54 system is a key regulator of the reproductive system. We examined the effect of intracerebroventricular (i.c.v.) and peripheral administration of the active kisspeptin fragment, kisspeptin-10, on circulating gonadotropins and total testosterone levels in adult male rats. The effect of kisspeptin-10 *in-vitro* on the release of hypothalamic peptides from hypothalamic explants and gonadotropins from anterior pituitary fragments was also determined. The i.c.v. administration of kisspeptin-10 dose-dependently increased plasma luteinizing hormone (LH) and increased plasma follicle stimulating hormone (FSH) and total testosterone at 60 mins post-injection. In a separate study investigating the time course of this response, i.c.v. administered kisspeptin-10 (3nmol) significantly increased plasma LH at 10, 20 and 60 mins, FSH at 60 mins and total testosterone at 20 and 60 mins post-injection. Kisspeptin-10 stimulated the release of luteinizing hormone releasing hormone (LHRH) from *in-vitro* hypothalamic explants. Peripheral administration of kisspeptin-10 increased plasma LH, FSH and total testosterone. However, doses of 100-1000nM kisspeptin-10 did not influence LH or FSH release from pituitary fragments *in-vitro.* Kisspeptin therefore potently stimulates the hypothalamic- pituitary-gonadal (HPG) axis. These effects are likely to be mediated via the hypothalamic LHRH system.

## Introduction

Kisspeptin is a 54 amino acid peptide encoded by the tumour suppressor gene KiSS-1 (1-4). Kisspeptin is thus also known as ‘metastin’ because of its antimetastatic properties (4,5). Using quantitative polymerase chain reaction, KiSS-1 mRNA expression has been demonstrated in the placenta and throughout the central nervous system (CNS), including the hypothalamus (3). Endogenous forms of kisspeptin 54, 14 and 13 amino acids in length have been isolated from human placenta. The common C terminal decapeptide shared by these forms, kisspeptin-10, is secreted by cultured human trophoblasts (6). In humans, circulating kisspeptin levels are 7000-fold higher than basal levels during the third trimester of pregnancy (7).

All kisspeptin fragments, including kisspeptin-10, have a similar affinity and efficacy for the previously orphan G-protein-coupled receptor, GPR54 (1). GPR54 was originally isolated from rat brain (8) and is highly expressed in the rat and human CNS and peripheral tissues (1,8). GPR54 receptor mRNA is expressed in several rat brain regions, with highest expression in the hypothalamus and amygdala. Within the hypothalamus, GPR54 mRNA is highly concentrated in the arcuate nucleus, the lateral hypothalamic area and the dorsomedial nucleus (8). In the periphery it is highly expressed in the pituitary, placenta and pancreas (1,3). Peripheral administration of kisspeptin-10 increases plasma oxytocin levels in female rats (1).

Recent reports suggest that the kisspeptin/GPR54 system is a key regulator of the reproductive system. GPR54 deficient (GPR54-/-) mice have abnormal sexual development and low circulating gonadotropin concentrations (9,10). In humans, GPR54 mutations have been shown to cause isolated hypogonadotropic hypogonadism in two different consanguineous families (10,11). GPR54 thus appears essential for normal gonadotropin secretion and the regulation of puberty.

To investigate the role of the kisspeptin/GPR54 system in the hypothalamic-pituitary gonadal axis (HPG) axis, we examined the effect of central and peripheral administration of kisspeptin-10 on circulating gonadotropins and total testosterone levels in adult male rats. During the preparation of this manuscript, two independent groups demonstrated findings in accord to those observed in the present study. Matsui *et al* revealed that subcutaneously (s.c.) administered kisspeptin-54 increases plasma gonadotropins in male and prepubertal female rats (12) and Gottsch *et al* showed that i.c.v. administration of kisspeptin-10 or kisspeptin-54 increases plasma LH and FSH in male mice (13). Here we investigate the time course of i.c.v. effects of kisspeptin-10 on circulating gonadotropins in another species, the male rat, additionally determining the effects on plasma testosterone levels. We have also investigated the time course of the effects on plasma gonadotropins and testosterone of intraperitoneal (i.p.) injections of kisspeptin-10 in the male rat, a different route of peripheral administration to that previously studied. In addition, to investigate whether the actions of kisspeptin-10 are mediated via the hypothalamic LHRH system, we have examined the effect of kisspeptin-10 on the release of LHRH from hypothalamic explants. We have also investigated the effects of kisspeptin-10 on gonadotropin secretion from anterior pituitary fragments.

GPR54 and kisspeptin mRNA have been found in several hypothalamic nuclei involved in appetite control. Several hormones and neuropeptides are involved in the regulation of both the HPG axis and energy balance. The absence of the adipocyte hormone leptin suppresses the HPG axis (14). Central administration of the hypothalamic neuropeptides galanin-like peptide (GALP) or prolactin releasing peptide (PrRP) influence both feeding behaviour and the gonadotropic axis (15-17).

We have therefore investigated the effect of i.c.v. administered kisspeptin-10 on food intake in three models of feeding behaviour.

## Methods

### Materials

Kisspeptin-10 was purchased from Peptide Institute Inc (Osaka, Japan). Reagents for basal hypothalamic explant experiments and pituitary fragments experiments were supplied by BDH (Poole, Dorset, UK).

### Animals

Male Wistar rats (Specific pathogen free, Charles River, UK) weighing 250-300g were maintained in individual cages for cannulation and similar rats weighing 200-250g were caged in groups of five for use in peripheral studies and in hypothalamic and pituitary explant experiments. All animals were maintained under controlled temperature (21-23$^{o}$C) and light (12 hrs light, 12 hrs dark, lights on at 07.00h) with *ad libitum* access to food (RM1 diet, SDS UK Ltd.) and water, unless otherwise specified.

All animal procedures were conducted under the British Home Office Animals Scientific Procedures Act 1986 (Project Licence 70/5516) and in accordance with accepted standards of the local ethical review committee.

### Intracerebroventricular cannulation and injections

Animals were implanted with permanent 22-gauge stainless steel i.c.v. cannulae projecting to the third cerebral ventricle (co-ordinates; 0.8mm posterior to bregma on the midline and implanted 6.5mm below the outer surface of the skull) as previously

described (18). Following a seven-day recovery period, the animals were acclimatised to the injection procedure and to the guillotine apparatus to minimise 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 (150ng/rat), were included in the studies.

All studies were carried out in the early light phase (08.00h – 11.00h) unless otherwise specified. Peptides were dissolved in 0.9% saline and administered in a 5µl volume via a stainless steel injector projecting 0.5mm beyond the tip of the cannula. The injector was connected by polyethylene tubing (id, 0.5 mm; od, 1 mm) to a Hamilton syringe (Reno, NV, USA) in a syringe pump set to dispense 5µl solution/minute.

**Study 1(i) Dose-response of i.c.v. kisspeptin-10 on the hypothalamic-pituitary-gonadal axis.**

Groups of rats (n =10-11/group) were i.c.v. injected with either 0.9% saline or kisspeptin-10 at 0.1, 0.3, 1 or 3nmol. At 60 mins following injection, rats were decapitated and trunk blood collected into lithium heparin tubes containing 0.6mg aprotinin (Bayer Corp., Haywards Heath, UK). Plasma was separated by centrifugation, frozen on dry ice and stored at –20$^{o}$C until measurement of LH, FSH and total testosterone.

**Effect of i.c.v. administration of kisspeptin-10 on behaviour**

Behaviour was observed for 1 hr in the saline- and 3nmol kisspeptin-treated groups. Rats (n = 7-8/group) were observed continuously for 1 hr post i.c.v. injection by observers blinded to the experimental treatment. Behaviour was classified, as

previously described, into seven different categories: feeding, drinking, grooming, rearing (defined as stationary with front paws elevated), head down (defined as stationary with all four paws on the cage bottom), locomotion (defined as moving around the cage, with all four paws moving), or sleeping (19). Each rat was observed for 15 sec every 5 min during the test session. This 15-sec period was further divided into three 5-sec periods, and the behaviour of the rat was scored in each section of the time period. Each rat had a total of 36 behaviours recorded per hr.

**Study 1(ii) Time course of effect of i.c.v. kisspeptin-10 on the hypothalamic-pituitary-gonadal axis.**

Groups of rats (n =8-10/group) were i.c.v. injected with either 0.9% saline or kisspeptin-10 (3nmol). At 10, 20 and 60 mins following injection, rats were decapitated, trunk blood collected and stored as above until measurement of LH, FSH and total testosterone. We have previously used 10, 20 and 60 minute time points to investigate changes in plasma LH and FSH following i.c.v. administration of prolactin-releasing peptide (20).

**Study 2. Effect of kisspeptin-10 on hypothalamic releasing hormones**

The static incubation system method used was as previously described (21). Male Wistar rats were killed by decapitation and the whole brain immediately removed. The brain was mounted with ventral surface uppermost and placed in a vibrating microtome (Microfield Scientific Ltd., Dartmouth, UK). A 1.7mm slice was taken from the basal hypothalamus, to include the medial preoptic area (MPOA), and incubated in individual chambers containing 1ml of artificial cerebrospinal fluid (aCSF), (20mM $NaHCO_3$, 126mM NaCl, 0.09mM $Na_2HPO_4$, 6mM KCl, 1.4mM

$CaCl_2$, 0.09mM $MgSO_4$, 5mM glucose, 0.18mg/ml ascorbic acid and 100ug/ml aprotinin) equilibrated with 95% $O_2$ and 5% $CO_2$. The tubes were placed on a platform in a water bath maintained at 37$^{o}$C. After an initial 2 hr equilibration period, the hypothalami were incubated for 45 mins in 600µl aCSF (basal period) before being incubated with kisspeptin-10 at 1, 10 or 100nM for 45 minute test period (n=13-22/dose). The viability of the tissue was verified by a final 45 minute exposure to 56mM KCl; isotonicity was maintained by substituting $K^+$ for $Na^+$. Explants not showing significant release following KCl (<10%) were excluded from the analysis. At the end of each period, the aCSF was removed and frozen at –20$^{o}$C until measurement of LHRH, oxytocin and galanin by radioimmunoassay (RIA). Oxytocin and galanin have been shown to be involved in reproductive function (22,23).

**Study 3. Effect of peripheral administration of kisspeptin-10 on the hypothalamic-pituitary-gonadal axis**

**(i)** Rats (n=9-11/group) received an intraperitoneal (i.p.) injection of 0.5ml 0.9% saline for three days to acclimatise them to the injection procedure and also underwent sham decapitation for 2 days prior to the study day. On the following day, rats were i.p. injected with either 0.9% saline, kisspeptin-10 at 10, 30, 100nmol, or 30nmol LHRH as a positive control. At 20 mins following injection, rats were decapitated and trunk blood was collected and stored as above until measurement of LH, FSH and total testosterone.

**(ii)** Rats (n=8-10/group) were i.p. injected as described above in 3(i). At 60 mins following injection, rats were decapitated, trunk blood collected and stored as above.

**(iii)** The results of study 3(ii) did not show an increase in plasma FSH that may have been expected following the i.c.v. studies. Study 3(ii) used i.p. injections of kisspeptin-10 up to 100nmol. To further investigate the effect of peripheral kisspeptin-10 on FSH, rats (n=9-11/group) were treated with saline or 300nmol kisspeptin-10 and decapitated at 60 min following injection. Trunk blood was collected and stored as above.

**Study 4. Effect of kisspeptin-10 on gonadotropin release from anterior pituitary fragments**

The effects of kisspeptin-10 on pituitary LH and FSH release were determined using anterior pituitary segments. The method was a modification of that previously described (24). Rats were decapitated and anterior pituitary glands were harvested immediately then divided into 4 pieces of approximately equal size. The segments were randomly placed (1 segment/well) in the wells of a 48-well tissue culture plate (Nunc International, Denmark) and incubated in 500µl of aCSF as detailed above. The anterior pituitary segments were maintained at 37$^{o}$C in a humidified environment saturated with 95% $O_2$ and 5% $CO_2$ for 2 hr with the medium changed after 1hr and 2hr. The segments were then incubated in aCSF alone (control), kisspeptin-10 at 100 or 1000nM, or 100nM LHRH as a positive control for 4hr (n=24/group). At the end of this period, the aCSF was collected and stored at –20$^{o}$C until RIA for LH. As no dose of kisspeptin-10 showed a significant effect on LH release, only the aCSF alone, kisspeptin-10 1000nM and LHRH 100nmol samples were assayed for FSH.

**Study 5(i). ICV kisspeptin-10 on food intake in fed animals during the early light phase**

Feeding experiments were carried out in i.c.v. cannulated rats as previously described (25). *Ad libitum* fed rats were i.c.v. injected between 08.30h and 09.30h with 0.9% saline, kisspeptin-10 at 0.3, 1, or 3nmol or 5nmol NPY (as a positive control) (n =8-11/group) . Rats were returned to their home cages with a pre-weighed amount of chow and free access to water. The remaining food was reweighed at 1, 2, 4, 8 and 24 hrs post injection. A dose of 3nmol kisspeptin-10 was the highest used as pilot studies showed higher doses (10nmol) produced adverse behavioural effects (data not shown).

**Study 5(ii). ICV kisspeptin-10 on food intake in fasted animals during the early light phase**

Rats fasted overnight were i.c.v. injected between 08.30h and 09.30h with 0.9% saline, kisspeptin-10 at 0.3, 1, or 3nmol or 3nmol NDP-alpha-melanocyte stimulating hormone (NDP-MSH) (as a positive control) (n=8-10/group) . Rats were returned to their home cages with a pre-weighed amount of chow and free access to water. The remaining food was reweighed at 1, 2, 4, 8 and 24 hrs post injection.

**Study 5 (iii). ICV kisspeptin-10 on food intake in fed animals during the early dark phase**

To check whether kisspeptin-10 effects food intake during the natural rat feeding period, a*d-libitum* fed rats were i.c.v. injected with 0.9% saline, kisspeptin-10 at 3nmol or 3nmol NDP-MSH, (as a positive control) immediately before the onset of dark phase (19.00h)(n=8-10/group). Rats were returned to their home cages with a pre-weighed amount of chow and free access to water. The remaining food was

reweighed at 1, 2, 4, 8 and 24 hrs post injection. Only 3nmol kisspeptin-10, the highest dose previously used, was administered in this study as no effect on appetite had been observed in 5(i) and 5(ii).

**Hormone assays**

LHRH was measured in aCSF by a sensitive and specific radioimmunoassay (RIA) (reagents and methods provided by Dr. H.M. Fraser, Medical Research Council Reproductive Biology Unit, Edinburgh, Scotland). Oxytocin and galanin were measured by RIA as previously described (26,27). LH levels in plasma were assayed using reagents and methods provided by the NIDDK and the National Hormone and Pituitary Program (Dr. A. Parlow, Harbor University of CA, Los Angeles Medical Center, USA) as previously described (28). The intra- and inter- assay coefficients of variation were 8.2% and 13.6% respectively. Plasma FSH was measured by commercial immuno-radiometric assay (IDS Ltd, Boldon, UK) and total plasma testosterone by commercial Coat-a-Count assay kit (EURO/DPC Limited, Caernarfon, UK).

**Statistical analysis**

Results are shown as mean values ± SEM. Data from hypothalamic explant release experiments were compared by paired *t-test* between the basal period and test period. Data from the i.c.v. time-course study was compared by unpaired *t-test* between saline and kisspeptin-10 groups at each time point. Data from study 3(iii) was compared by unpaired *t-test* between saline and 300nmol kisspeptin-10 at the 60 minute time point. As behavioural observation data were not normally distributed, Kruskal-Wallis test was used for comparisons between treatment groups. All other *in vivo* and *in vitro*

study results were compared by analysis of variance (ANOVA) with *post hoc* Dunnett's test (Systat, Evanston, IL). In all cases, $p<0.05$ was considered to be statistically significant.

## Results

### Study 1(i) Dose response of i.c.v. kisspeptin-10 on the hypothalamic-pituitary-gonadal axis.

Intracerebroventricular administration of kisspeptin-10 dose-dependently increased plasma LH and increased plasma FSH and testosterone 60 mins post injection. Plasma LH was significantly increased following i.c.v. injection of 1 and 3nmol kisspeptin-10 (saline 0.3 ± 0.02ng/ml; 1nmol kisspeptin 5.0 ± 1.17ng/ml, 3nmol kisspeptin 9.4 ± 1.85ng/ml, $p<0.05$, n=9-11/group) (Figure 1(i)A). Plasma FSH was significantly increased following i.c.v. injection of 1nmol kisspeptin-10 (saline 13.2 ± 1.45ng/ml; 1nmol kisspeptin 23.8 ± 3.08ng/ml $p<0.05$, n=9-11/group). Plasma FSH was increased following i.c.v. injection of 0.3nmol and 3nmol kisspeptin-10 (0.3nmol kisspeptin 20.8 ± 3.35ng/ml, 3nmol kisspeptin 20.2 ± 2.9ng/ml), however this did not reach significance (Figure 1(i)B). Total plasma testosterone was significantly increased following i.c.v. injection of 0.1, 0.3, 1, and 3nmol kisspeptin-10 (saline 5.8 ± 0.72nmol/L; 0.1nmol kisspeptin 15.7 ± 2.98nmol/L, 0.3nmol kisspeptin 13.8 ± 3.02nmol/L, 1nmol kisspeptin 21.9 ± 1.46nmol/L, 3nmol kisspeptin 21.2 ± 1.16nmol/L, $p<0.05$, n=9-11/group) (Figure 1(i)C). No significant differences in behaviour were observed following i.c.v. injection of 3nmol kisspeptin-10 over the 1 hr observation period (Table 1).

**Study 1(ii). Time course of i.c.v. kisspeptin-10 on the hypothalamic-pituitary-gonadal axis.**

Intracerebroventricular injection of 3nmol kisspeptin-10 significantly increased plasma LH at 10 mins (saline 1.0 ± 0.08ng/ml; kisspeptin 1.7 ± 0.29ng/ml, $p<0.05$, n=9-10/group) and this effect was maintained at 20 mins (saline 0.8 ± 0.08ng/ml; kisspeptin 2.7 ± 0.48ng/ml, $p<0.01$, n=8-10/group) and 60 mins (saline 0.3 ± 0.02ng/ml; kisspeptin 5.2 ± 1.13ng/ml, $p<0.001$, n=8-10/group) post-injection (Figure 1(ii)A). Plasma FSH was significantly increased at 60 mins post-injection (saline 16.9 ± 1.46ng/ml; kisspeptin 34.4 ± 3.71ng/ml, $p<0.001$, n=8-10/group) (Figure 1(ii)B). In the previous i.c.v. dose response study, 3nmol kisspeptin-10 increased plasma FSH at 60 mins, but this change did not reach statistical significance. In this experiment the rise is statistically significant. This discrepancy may be due to variation between the different groups of animals or assay variation. Total plasma testosterone was significantly increased at 20 and 60 mins following injection (20 mins: saline 3.0 ± 0.27nmol/L; kisspeptin 6.4 ± 1.19nmol/L, $p<0.05$, n=8-10/group; 60mins: saline 4.1 ± 0.36 nmol/L; kisspeptin 14.2 ± 2.80nmol/L, $p<0.01$, n=8-10/group) (Figure 1(ii)C).

**Study 2. Effect of kisspeptin-10 on hypothalamic releasing hormones**

Administration of kisspeptin-10 to hypothalamic explants stimulated the release of LHRH at doses of 10 and 100nM (Figure 2). Kisspeptin did not significantly alter oxytocin or galanin release from hypothalamic explants (Table 2).

**Study 3. Effect of peripheral administration of kisspeptin-10 on the hypothalamic-pituitary-gonadal axis**

**(i)** Peripheral injection of 100nmol kisspeptin-10 significantly increased plasma LH at 20 mins post injection (saline 0.6 ± 0.06ng/ml; 100nmol kisspeptin 5.9 ± 1.55ng/ml, $p<0.05$, n=9-11/group) (Figure 3(i)A). Injection of 10 and 30nmol kisspeptin-10 also increased plasma LH, but these changes did not reach statistical significance. There was no effect on plasma FSH and total testosterone 20 mins post injection (Figures 3(i) B & C).

**(ii)** Peripheral injection of 10, 30 and 100nmol kisspeptin-10 did not increase plasma LH or FSH at 60 mins post-injection (Figure 3(ii) A & B). Similarly, peripheral administration of 30nmol LHRH did not significantly increase plasma LH or FSH at 60 mins post injection. However, 30 and 100nmol kisspeptin-10 and 30nmol LHRH did significantly increase total plasma testosterone at 60 mins post injection (saline 1.1 ± 0.39nmol/L; 30nmol kisspeptin 5.9 ± 1.21nmol/L, 100nmol kisspeptin 5.8 ± 1.43nmol/L, 30nmol LHRH 5.8 ± 1.26nmol/L, $p<0.05$, n=8-10/group). The rise in total plasma testosterone produced by 30 and 100nmol kisspeptin-10 is of a similar magnitude to that induced by 30nmol LHRH (a 6-fold increase from saline control). (Figure 3(ii)C).

**(iii)** Peripheral injection of 300nmol kisspeptin-10 significantly increased plasma LH, FSH and total testosterone at 60 mins post injection (LH: saline 0.5 ± 0.08ng/ml; 300nmol kisspeptin 0.9 ± 0.16ng/ml, $p<0.05$. FSH: saline 12.9 ± 0.96ng/ml; 300nmol kisspeptin 16.9 ± 1.45ng/ml, $p<0.05$. Total testosterone: saline 3.4 ± 0.37nmol/L; 300nmol kisspeptin 16.7 ± 6.18nmol/L, $p<0.05$, n=9-10/group).

**Study 4. Effect of kisspeptin-10 on gonadotropin release from anterior pituitary fragments**

Kisspeptin-10 at doses 100 and 1000nM had no effect on the release of LH or FSH from *in-vitro* pituitary fragments (Table 3). This study was repeated twice.

**Study 5 (i). Effect of ICV kisspeptin-10 on food intake in fed animals during the early light phase**

There were no differences in food intake between saline- and kisspeptin-treated groups at 1, 2, 4, 8 and 24 hrs post injection (Food intake at 0-2 hr shown in Figure 4A). The positive control, 5nmol NPY, significantly increased food intake at 0-2 hrs. There were no differences in body weight change between saline and kisspeptin groups over the 24 hr period (Data not shown).

**Study 5(ii). ICV kisspeptin-10 on food intake in fasted animals during the early light phase**

There were no differences in food intake between saline- and kisspeptin-treated groups at 1, 2, 4, 8 and 24 hrs post injection (Food intake at 0-2 hr shown in Figure 4B). The positive control, 3nmol NDP-MSH significantly decreased food intake at 0-2 hrs. There were no differences in body weight change between saline and kisspeptin groups over the 24 hr period (Data not shown).

**Study 5(iii). ICV kisspeptin-10 on food intake in fed animals during the early dark phase**

There were no differences in food intake between saline- and kisspeptin-treated groups at 1, 2, 4, 8 and 24 hrs post injection (Food intake at 0-2 hrs shown in Figure 4C). The positive control, 3nmol NDP-MSH significantly decreased food intake at 0-

2 hrs. There were no differences in body weight change between saline and kisspeptin groups over the 24 hr period (Data not shown).

**Discussion**

We have demonstrated that kisspeptin-10, a fragment of the endogenous agonist for the GPR54 receptor, stimulates the HPG axis. Both i.c.v. and peripheral administration of kisspeptin-10 increased plasma LH, FSH and total testosterone.

During the preparation of this manuscript, two reports were published which showed similar effects of kisspeptin on the HPG axis in accord with our own observations (12,13). We have shown kisspeptin-10 dose-dependently stimulates the release of LHRH from *in-vitro* hypothalamic explants, suggesting that kisspeptin stimulates the HPG axis via LHRH. Current evidence certainly suggests that kisspeptin operates upstream of LHRH. Female GPR54-/- mice can be induced to ovulate by exogenous administration of LHRH (10). Humans with GPR54 mutations have a suppressed HPG axis which is responsive to pulsatile administration of exogenous LHRH (10). The i.c.v. and peripheral actions of kisspeptin can be blocked by LHRH antagonists (12,13). It is possible that i.c.v. kisspeptin-10 influences intermediate neurons which regulate LHRH neurons. However, the GPR54 receptor has been localised to hypothalamic LHRH neurons in cichlid fish, suggesting a direct effect of kisspeptin on these neurons (29).

GPR54 is expressed in the pituitary gland (1,3). It is possible peripherally administered kisspeptin acts directly on the pituitary to stimulate the release of LH and FSH. However, in the present study, kisspeptin had no effect on the release of LH

or FSH from male rat anterior pituitary fragments, while LHRH significantly increased LH and FSH release. GPR54 mRNA is also highly expressed within the arcuate nucleus, an area accessible to circulating hormones (8,30). The stimulation of the HPG axis seen following peripheral administration of kisspeptin may therefore be centrally mediated, possibly via GPR54 receptors in the arcuate nucleus. Experiments to investigate whether circulating kisspeptin enters the hypothalamus are required to further elucidate the mechanism of action of peripheral kisspeptin.

Plasma LH levels were highest 60 mins following central administration of kisspeptin. However, following i.p. kisspeptin administration plasma LH levels were highest 20 mins following injection and by 60 mins post injection only the highest kisspeptin dose (300nmol) caused any increase in plasma LH. Circulating kisspeptin may be degraded or cleared more rapidly than central kisspeptin thus reducing the duration of its effects. In contrast to our study, Matsui *et al*, observed raised plasma LH levels up to 2 hrs following peripheral kisspeptin administration (12). Matsui *et al* administered full length kisspeptin-54 subcutaneously. We have shown that the effects of i.p. kisspeptin-10 on LH release are short lived. This may be because kisspeptin-10 is inactivated or cleared more rapidly than kisspeptin-54. It is also possible that peripheral kisspeptin only has a short lived effect and the more sustained rise in LH seen with subcutaneous administration is due to a gradual release of peptide into the circulation from a subcutaneous depot. Further investigations into the pharmacokinetics of centrally and peripherally administered kisspeptins are required.

GPR54 and kisspeptin mRNA have been detected in several hypothalamic nuclei involved in regulation of feeding. GPR54 is expressed in the arcuate nucleus, lateral

hypothalamus, and the dorsomedial nucleus (8), and kisspeptin in the arcuate nucleus and the paraventricular nucleus (PVN) (13). We therefore studied the effects of acute i.c.v. administration of kisspeptin-10 on food intake in rats in three models: *ad libitum* fed rats in the early light phase, overnight fasted rats in the early light phase, and in *ad libitum* fed rats in the early dark phase. In all three models, kisspeptin-10 had no effect on food intake at any of the doses or time points studied (1, 2, 4, 8 and 24 hrs post injection). The highest i.c.v. dose of kisspeptin-10 (3nmol) had no effect on behaviour. Analysis of in behaviour is used to investigate obvious adverse effects. For example, an increase in grooming is often associated with the activation of central stress pathways (31). In addition, the physiological significance of any effect on appetite can be assessed by comparing changes to the Behavioural Satiety Sequence (32), a model describing observed changes in behaviour following normal satiation. Chemicals/peptides can suppress eating by producing adverse physiological effects such as pain, or illness, or may increase food intake as a secondary effect of increased arousal. We have found that centrally administered kisspeptin-10 does not appear to influence locomotive or sleeping behaviour and also has no effect on food intake or feeding behaviour. Hypothalamic kisspeptin seems unlikely to play a major role in the regulation of appetite.

In summary, we have described the effects of kisspeptin-10 on the HPG axis. Both central and peripheral administration of kisspeptin-10 increase plasma LH, FSH and total testosterone. Kisspeptin-10 stimulated the release of LHRH from hypothalamic explants. However, kisspeptin-10 had no effect on LH or FSH release from pituitary fragments, suggesting that peripheral kisspeptin stimulates the HPG axis via the hypothalamus. Further studies are required to define the physiological importance of

kisspeptin in the regulation of the HPG axis. The chronic effects of kisspeptin administration on the HPG axis and possible changes in central and peripheral kisspeptin expression in models of disrupted HPG activity would be particularly interesting to investigate.

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**Figure Legends**

**Figure 1(i).** The effect of i.c.v. saline (0) or kisspeptin-10 (0.1, 0.3, 1, 3nmol) on plasma levels of (**A**) LH, (**B**) FSH and (**C**) total testosterone in male Wistar rats at 60 mins post injection. Significance is indicated by * $p<0.05$ vs saline control. n=9-11/group.

**Figure 1(ii).** The effect of i.c.v. saline or kisspeptin-10 (3nmol) at 10, 20 and 60mins following injection on plasma levels of (**A**) LH, (**B**) FSH and (**C**) total testosterone in male Wistar rats. Striped bar indicates saline and hatched bar indicates kisspeptin. Significance is indicated by * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs saline control. n=8-10/group.

**Figure 2.** Kisspeptin-10 (1nM, 10nM, 100nM) stimulation of basal LHRH secretion from *in-vitro* hypothalamic explants. The positive control was aCSF containing 56nM $K^+$. Significance is indicated by * $p<0.05$, ***$p< 0.001$ vs aCSF control. n=16-22/group.

**Figure 3(i).** The effect of peripheral (i.p.) saline (0), kisspeptin-10 (10, 30, 100nmol) or 30nmol LHRH at 20 mins following injection on plasma levels of (**A**) LH, (**B**) FSH and (**C**) total testosterone in male Wistar rats. Significance is indicated by * $p<0.05$ vs saline control. n= 9-11/group.

**Figure 3(ii).** The effect of peripheral (i.p.) saline (0), kisspeptin-10 (10, 30, 100nmol) or 30nmol LHRH at 60 mins following injection on plasma levels of (**A**) LH, (**B**) FSH

and (**C**) total testosterone in male Wistar rats. Significance is indicated by * $p<0.05$ vs saline control. n= 8-10/group.

**Figure 4. (A)** The effects of i.c.v. kisspeptin-10 (0.3, 1, 3nmol), saline (control) or NPY (5nmol) on food intake in *ad libitum* fed rats in the early light phase in the first 2 hrs following injection. n= 8-11/group. **(B)** The effects of i.c.v. kisspeptin-10 (0.3, 1, 3nmol), saline (control) or NPD-MSH (3nmol) on food intake in overnight fasted rats in the early phase in the first 2 hrs following injection. n=8-10/group. **(C)** The effects of i.c.v. kisspeptin-10 (3nmol), saline (control) or NDP-MSH (3nmol) on food intake in *ad libitum* fed rats in the early dark phase in the first 2 hrs following injection. n=8-10/group. Significance is indicated by *$P < 0.05$.

**Table 1**

Behaviour over the first 1 hr following i.c.v. injection of kisspeptin-10 (3nmol) or saline.

| **% of time spent in a behaviour (interquartile range)** | | |
|---|---|---|
| | **Saline** | **Kisspeptin (3nmol)** |
| **Feeding** | 0 (0- 8.3) | 0 (0- 6.3) |
| **Drinking** | 0 (0- 0) | 0 (0- 0) |
| **Grooming** | 13.9 (8.3- 16.7) | 2.8 (0- 9.0) |
| **Rearing** | 19.4 (8.3- 22.2) | 5.6 (2.8- 13.2) |
| **Locomotion** | 5.6 (5.6- 9.7) | 12.5 (9.7- 15.3) |
| **Head down** | 16.7 (11.1- 22.2) | 16.7 (10.4- 18.1) |
| **Sleeping** | 50 (33.3- 50) | 50 (37.5- 70.1) |

Rats (n=7-8/group) were i.c.v. injected with 3nmol kisspeptin-10 or saline. Results are expressed as the median percentage (interquartile range) of the total time of observation spent in a particular behaviour.

**Table 2**

The effect of kisspeptin-10 on release of peptides from *in-vitro* hypothalamic explants.

| | **Basal** | **Kisspeptin 1nM** | **Kisspeptin 10nM** | **Kisspeptin 100nM** | **Potassium 56mM** |
|---|---|---|---|---|---|
| **Oxytocin** | 100 (± 13) | 105 (± 29) | 81 (± 26) | 131 (± 22) | 222 (± 10) |
| **Galanin** | 100 (± 13) | 81 (±14) | 97.6 (± 17) | 101 (± 22) | 120 (± 14) |

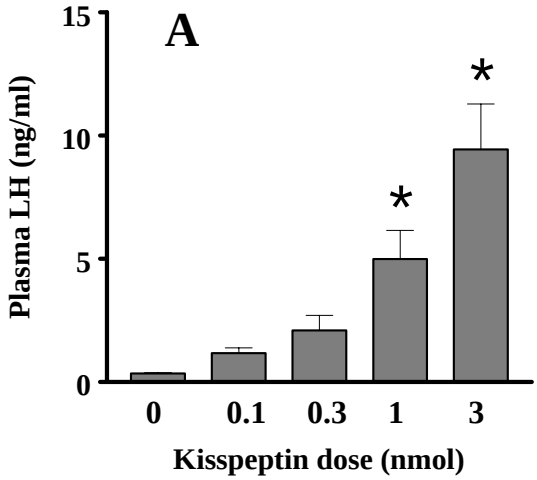
All values are expressed as a percentage of the basal release ± SEM, n=13-22/group.

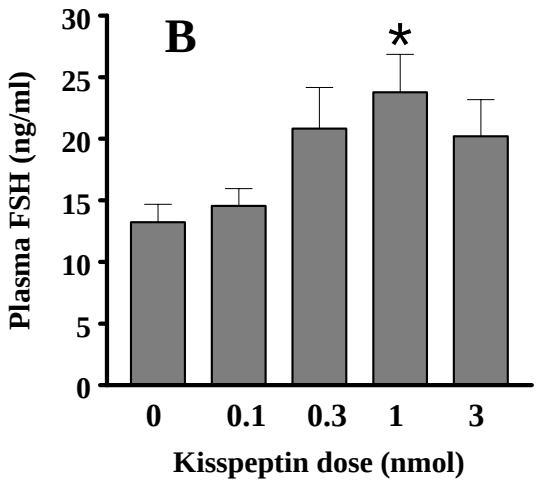
**Table 3**

The effect of kisspeptin-10 on the release of gonadotropins from *in-vitro* anterior pituitary fragments.

| | **Basal** | **Kisspeptin 100nmol** | **Kisspeptin 1000nmol** | **LHRH 100nmol** |
|---|---|---|---|---|
| **LH** | 100 (± 11.1) | 95 (± 7.4) | 96 (± 7.7) | 188.5 ******* (± 8.3) |
| **FSH** | 100 (± 8.4) | -------- | 88 (± 6.7) | 146 ***** (± 9.8) |

All values are expressed as a percentage of the basal release ± SEM. Significance is indicated by * $p<0.05$, *** $p<0.001$ vs basal release. n=24/group


A
Plasma LH (ng/ml)
15
10
5
0
*
*
0
0.1
0.3
1
3
Kisspeptin dose (nmol)


B
Plasma FSH (ng/ml)
30
25
20
15
10
5
0
*
0
0.1
0.3
1
3
Kisspeptin dose (nmol)

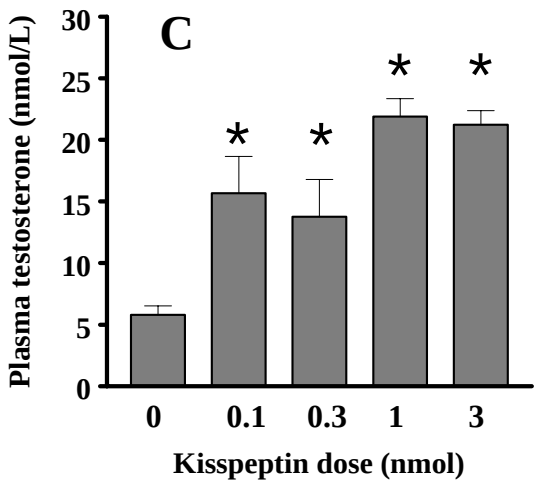

C
Plasma testosterone (nmol/L)
30
25
20
15
10
5
0
*
*
*
*
0
0.1
0.3
1
3
Kisspeptin dose (nmol)

Fig 1(i).

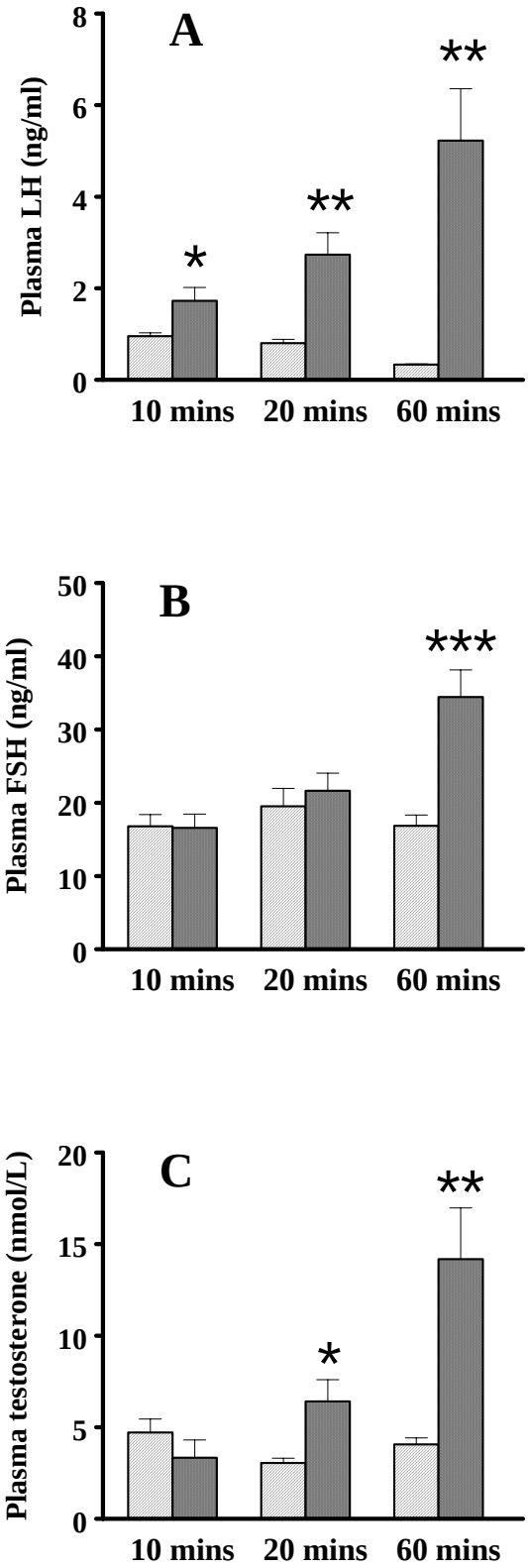

A
Plasma LH (ng/ml)
8
6
4
2
0
*
**
**
10 mins
20 mins
60 mins
B
Plasma FSH (ng/ml)
50
40
30
20
10
0
***
10 mins
20 mins
60 mins
C
Plasma testosterone (nmol/L)
20
15
10
5
0
*
**
10 mins
20 mins
60 mins

Fig 1(ii)

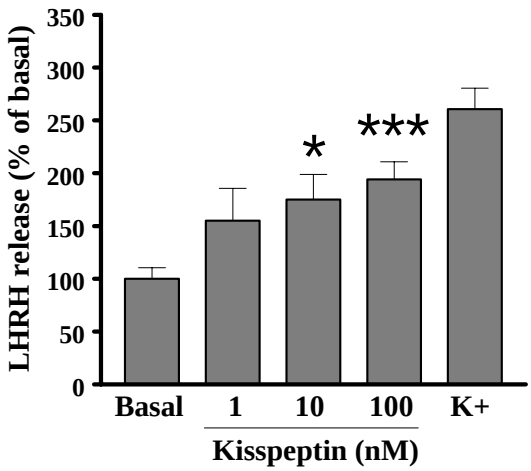
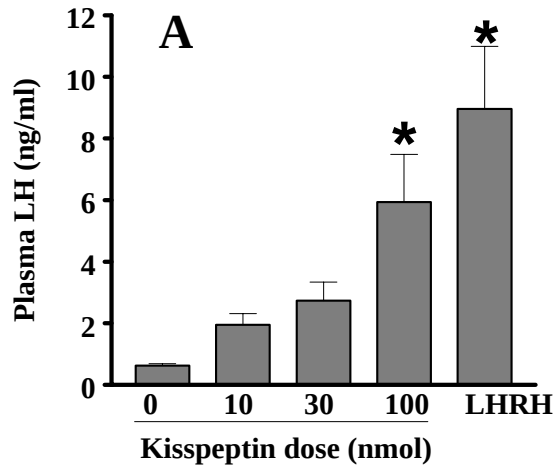
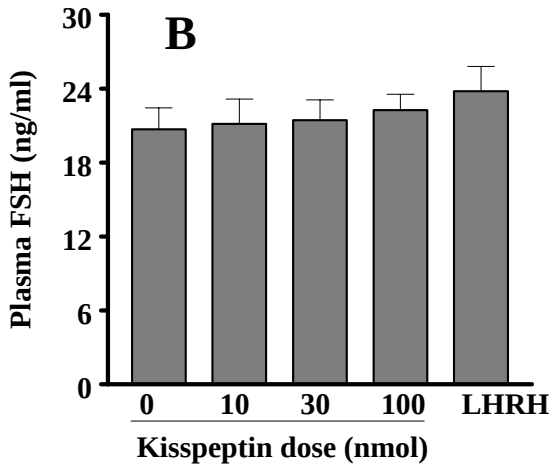

LHRH release (% of basal)
350
300
250
200
150
100
50
0
*
***
Basal
1
10
100
K+
Kisspeptin (nM)

Fig 2.


A
Plasma LH (ng/ml)
12
10
8
6
4
2
0
*
*
0
10
30
100
LHRH
Kisspeptin dose (nmol)


B
Plasma FSH (ng/ml)
30
24
18
12
6
0
0
10
30
100
LHRH
Kisspeptin dose (nmol)

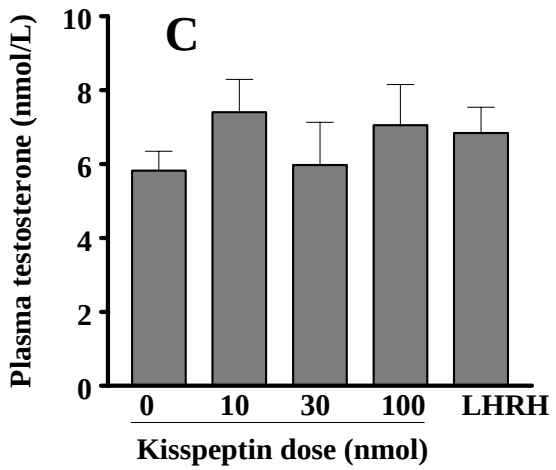
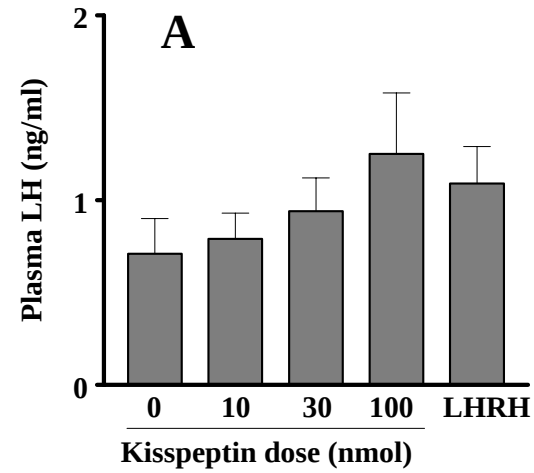
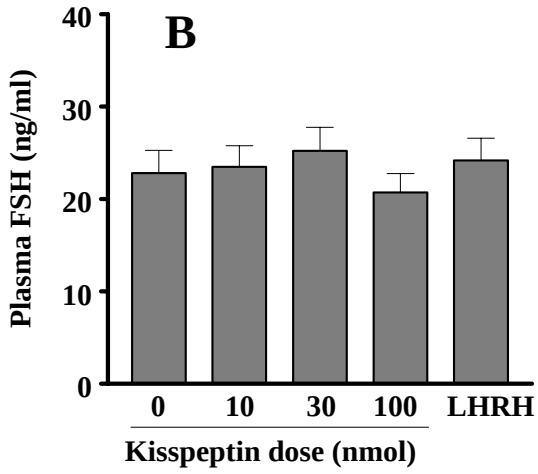

C
Plasma testosterone (nmol/L)
10
8
6
4
2
0
0
10
30
100
LHRH
Kisspeptin dose (nmol)

Fig 3(i)


A
Plasma LH (ng/ml)
2
1
0
0
10
30
100
LHRH
Kisspeptin dose (nmol)


B
Plasma FSH (ng/ml)
40
30
20
10
0
0
10
30
100
LHRH
Kisspeptin dose (nmol)

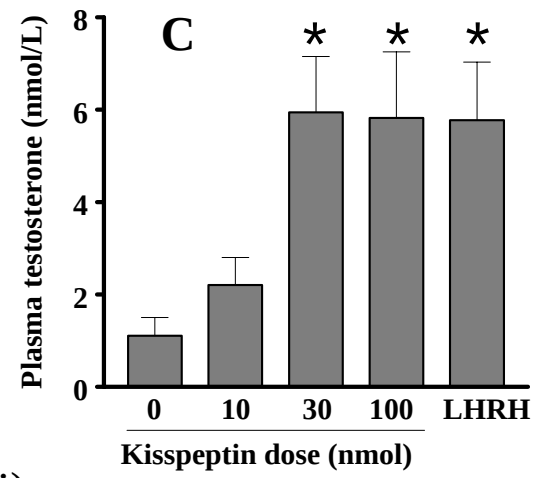
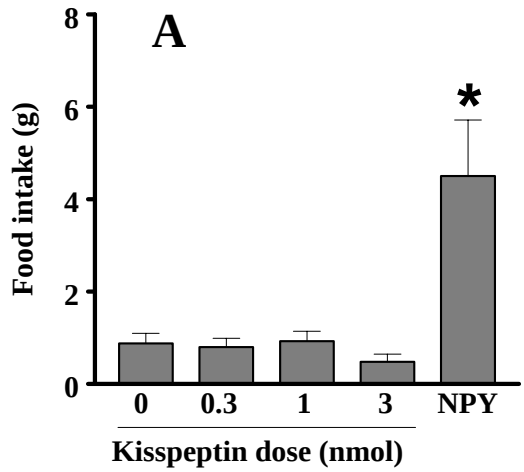
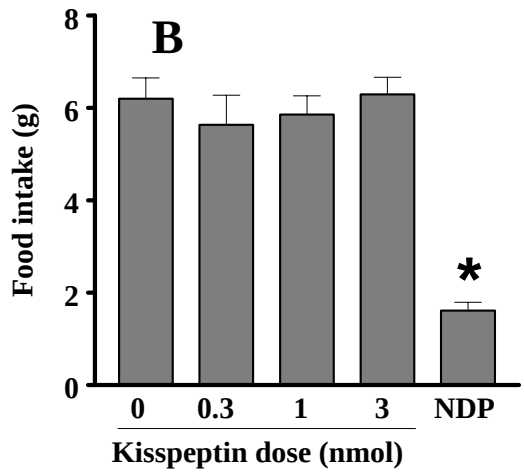

C
*
*
*
Plasma testosterone (nmol/L)
8
6
4
2
0
0
10
30
100
LHRH
Kisspeptin dose (nmol)

Fig 3(ii)


A
Food intake (g)
8
6
4
2
0
*
0
0.3
1
3
NPY
Kisspeptin dose (nmol)


B
Food intake (g)
8
6
4
2
0
*
0
0.3
1
3
NDP
Kisspeptin dose (nmol)

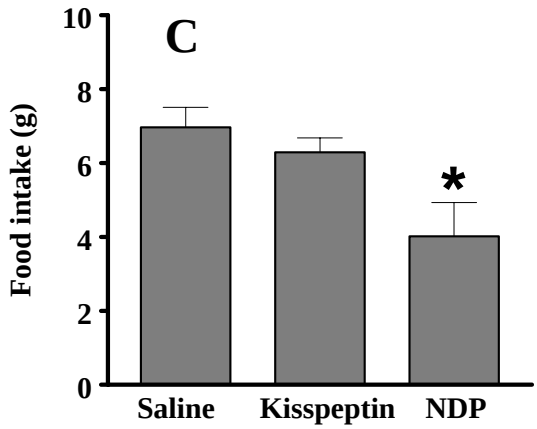

C
Food intake (g)
10
8
6
4
2
0
*
Saline
Kisspeptin
NDP

Fig 4