Investigation of the novel hormone kisspeptin in disorders of reproduction

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Abstract

The novel hormone kisspeptin has been identified to play a pivotal role in the regulation of the hypothalamo-pituitary-gonadal axis. Previous studies from our laboratory have demonstrated that a bolus administration of kisspeptin-54 can acutely stimulate gonadotrophin release in healthy men and women. However, no previous studies have examined the effects of kisspeptin-54 to women with infertility. In this study I have examined the effects of acute and chronic administration of kisspeptin-54 on women with infertility due to hypothalamic amenorrhoea. This study has identified that acute administration of kisspeptin to women with hypothalamic amenorrhoea results in stimulation of reproductive hormones, but chronic administration for two weeks of twice daily injections of kisspeptin-54 at a dose of 6.4 nmol/kg, results in tachyphylaxis. I have conducted further studies to examine the time course of desensitisation of the kisspeptin receptor. I have also determined that a dosing regime of twice weekly administration of kisspeptin-54 results in sustained stimulation of gonadotrophin release. I have performed the first study of kisspeptin-10 administration to men and women. These results demonstrate that kisspeptin-10 stimulates gonadotrophin release in men as well as women during the preovulatory phase of the menstrual cycle; but fails to stimulate gonadotrophin release in women during the follicular phase. This reveals a sexual dimorphism in response to the kisspeptin-10. These findings have important ramifications for the potential use of kisspeptin-54 and kisspeptin-10 as a therapeutic agent in disorders of reproduction.
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<td>AN</td>
<td>anorexia nervosa</td>
</tr>
<tr>
<td>ARC</td>
<td>arcuate nucleus</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>AVPV</td>
<td>anteroventral periventricular nucleus of hypothalamus</td>
</tr>
<tr>
<td>BP</td>
<td>blood pressure</td>
</tr>
<tr>
<td>CRH</td>
<td>corticotrophin releasing hormone</td>
</tr>
<tr>
<td>DBP</td>
<td>diastolic blood pressure</td>
</tr>
<tr>
<td>ER</td>
<td>oestrogen receptor</td>
</tr>
<tr>
<td>FSH</td>
<td>follicular stimulating hormone</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GAD</td>
<td>glutamic acid decarboxylase</td>
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<tr>
<td>GnIH</td>
<td>gonadotrophin inhibitory hormone</td>
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<tr>
<td>GnRH</td>
<td>gonadotrophin releasing hormone</td>
</tr>
<tr>
<td>GPR54</td>
<td>G-protein coupled receptor 54</td>
</tr>
<tr>
<td>HA</td>
<td>hypothalamic amenorrhoea</td>
</tr>
<tr>
<td>HPG</td>
<td>hypothalamo-pituitary-gonadal</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>E2</td>
<td>oestradiol</td>
</tr>
<tr>
<td>FEI</td>
<td>free oestradiol index</td>
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<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>HR</td>
<td>heart rate</td>
</tr>
<tr>
<td>IHH</td>
<td>idiopathic hypogonadotrophic hypogonadism</td>
</tr>
<tr>
<td>icv</td>
<td>intracerebroventricular</td>
</tr>
<tr>
<td>iv</td>
<td>intravenous</td>
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<tr>
<td>ivb</td>
<td>intravenous bolus</td>
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<tr>
<td>IVF</td>
<td>in vitro fertilisation</td>
</tr>
<tr>
<td>IR</td>
<td>immunoreactivity</td>
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<tr>
<td>KP54</td>
<td>kisspeptin-54</td>
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<tr>
<td>KISS1R</td>
<td>kisspeptin receptor</td>
</tr>
<tr>
<td>LH</td>
<td>luteinising hormone</td>
</tr>
<tr>
<td>MBH</td>
<td>mediobasal hypothalamus</td>
</tr>
<tr>
<td>MCH</td>
<td>melanin-concentrating hormone</td>
</tr>
<tr>
<td>MMP2</td>
<td>matrix metalloprotease 2</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin protein</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl D,L-aspartate</td>
</tr>
<tr>
<td>NPY</td>
<td>neuropeptide tyrosine / neuropeptide Y</td>
</tr>
<tr>
<td>OCP</td>
<td>oral contraceptive pill</td>
</tr>
<tr>
<td>OHSS</td>
<td>ovarian hyperstimulation syndrome</td>
</tr>
<tr>
<td>POA</td>
<td>preoptic area</td>
</tr>
<tr>
<td>POMC</td>
<td>pro-opiomelanocortin</td>
</tr>
<tr>
<td>PYY</td>
<td>peptide tyrosine-tyrosine / peptide YY</td>
</tr>
<tr>
<td>RFRP</td>
<td>RF-related peptide</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>SBP</td>
<td>systolic blood pressure</td>
</tr>
<tr>
<td>sc</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>SHBG</td>
<td>sex hormone binding globulin</td>
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Declaration of contributors

Declaration of originality: The work described in this thesis is my own. Any collaboration and assistance is described below. Contributors are within Section of Investigative Medicine, Imperial College London unless stated otherwise

Chapter 2: The study protocol was designed by Professor. W.S. Dhillo. I recruited and obtained consent from all subjects with the assistance of Drs. C. Jayasena and A. Ranger. I performed these studies with the assistance of Drs. C. Jayasena and A. Ranger, V. Salem and R. Ramachandran. Drs. K.G. Murphy and O.B. Chaudhri aliquoted and tested the kisspeptin-54 peptide for bioactivity and toxicity. Histological analysis during toxicity analysis was performed by Prof. G. Stamp (Department of Histopathology, Imperial College London). Ultrasound examinations were performed by Dr. A. Lim and Mrs. D. Patel (Imaging Department, Charing Cross Hospital, Imperial College Healthcare NHS Trust). Screening MRI scans were performed by Ms. C. Todd and Dr. A. Mehta Patel (Imaging Department, Charing Cross Hospital, Imperial College Healthcare NHS Trust). All serum samples were analysed by myself (Department of Biochemistry, Charing Cross Hospital, Imperial College Healthcare NHS Trust). I performed all radioimmunoassays under the guidance of Prof. M.A. Ghatei.

Chapter 3: The study protocol was designed by Professor. W.S. Dhillo, Dr C. Jayasena and I. I recruited and obtained consent from all subjects with the assistance of Drs. C. Jayasena, S. Hameed and S. Zac-Varghese. I performed these studies with the assistance of Drs. C. Jayasena, and A Abbara. Dr. K.G. Murphy assisted me with aliquoting of kisspeptin-54 peptide. Bioactivity and toxicity testing of peptide, ultrasound and MRI scans, and serum analyses were performed as in Chapter 2.

Chapter 4: The study protocol was designed by Professor. W.S Dhillo, Dr C. Jayasena and I. I recruited, obtained consent from subjects with the assistance of Drs. C. Jayasena, A. Abbara, and A. Comninos. I performed these studies with the assistance of Drs. C.
Jayasena, A. Comninos, A. Abbara, A. Januszewki, L. Sriskandarajah L and Ms M Vaal and Ms Z Farzad. I performed all radio-immunoassays with Dr C. Jayasena under the guidance of Prof. M.A. Ghatei. All serum samples were analysed by myself (Department of Biochemistry, Charing Cross Hospital, Imperial College Healthcare NHS Trust).
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I would like to thank my family for their support and love throughout, especially my husband Rumant and children Manraj and Baby Grewal.

This thesis is dedicated to the memory of my mother Mrs. Jasbir Kaur Nijher.
Chapter 1

General Introduction
1.1 The hypothalamo-pituitary-gonadal axis

The regulation of reproductive function is controlled by the hypothalamo-pituitary-gonadal (HPG) axis. A pulsatile secretion of gonadotrophin releasing hormone (GnRH), a ten amino acid peptide (Figure 1), synthesized in hypothalamic neurons situated in the preoptic area (POA) and anterior hypothalamus, stimulates gonadotrophs via GnRH receptors in the anterior pituitary gland to release luteinizing hormone (LH) and follicle stimulating hormone (FSH) into the circulation (Figure 2) (Gore 2002). The secretion of LH also demonstrates a pulsatile pattern and reflects the pulsatile secretion of GnRH (Dierschke et al. 1970). LH and FSH in turn act on the gonads to activate gametogenesis and sex steroid production. Sex steroids and gonadotrophins inhibit further GnRH secretion, in a negative feedback system. A failure at any point in the HPG axis or metabolic stress and extremes in energy reserves may result in disordered reproductive function and infertility (Neill J.D 2006).

One in seven couples in the United Kingdom suffers with infertility (HEFA Fertility Facts and Figures 2008). The resulting impact on physical, psychological and social functioning can be dramatic. Current therapies for infertility are associated with limitations both in terms of side effects and drug delivery. Improving our understanding of the physiology of the HPG axis may lead to the development of novel therapies for infertility.

Figure 1. The amino acid sequence of GnRH decapetide.

pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂
Figure 2. The hypothalamic pituitary gonadal (HPG) axis (Conn & Ulloa-Aguirre 2010).

The HPG axis is controlled in a classical endocrine feedback loop. Gonadotrophin releasing hormone (GnRH) is synthesised and secreted by specialised neurons located in the preoptic and arcuate nucleus of the hypothalamus. GnRH neurons have projections to the median eminence (ME) where GnRH is released into the hypophyseal-portal capillary circulation to act on gonadotrope cells of the adenohypophysis (AH). This stimulates the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH), which in turn act on the gonads to activate gametogenesis and sex steroid production. The pulsatile release of GnRH, LH, and FSH are positively or negatively regulated by several hypothalamic neurotransmitters and sex steroids.
In the male, LH binds to LH receptors on the Leydig cells of the testis resulting in the stimulation of the synthesis and secretion of androgens, primarily testosterone. Testosterone stimulates spermatogenesis and the development of secondary sexual characteristics. In the female, LH stimulates secretion of testosterone from the theca cells of the ovary; this is converted into oestrogens by neighbouring granulosa cells. Follicle stimulating hormone (FSH) stimulates folliculogenesis. A mid-cycle surge of LH in the pre-ovulatory phase of the menstrual cycle induces ovulation (Levi-Setti et al. 2004). Although generally LH release is suppressed by negative feedback from ovarian steroids, during the pre-ovulatory phase the high levels of oestradiol secreted from the developing ovarian follicle and progesterone result in positive feedback and stimulate LH release. Following ovulation the corpus luteum forms in the follicle, which secretes progesterone and oestradiol. Throughout the menstrual cycle the changing levels of LH and FSH (figure 3) act in a synergistic manner to promote follicular development, oestradiol synthesis, follicular rupture and ovulation (Levi-Setti et al. 2004)
Figure 3. The human female menstrual cycle (Aitken et al. 2008).

The follicular phase of the menstrual cycle begins on the first day of menstrual bleeding. A decrease in levels of oestrogen and progesterone results in the breakdown and shedding of the endometrium. Follicle stimulating hormone (FSH) levels increase, stimulating the development of several oocyte follicles. FSH levels subsequently decrease and only one or two follicles continue to develop. The developing follicles release oestrogen, which initiates thickening of the endometrium. The second phase of the menstrual cycle is the ovulatory phase which begins at approximately day 13, levels of LH and FSH increase dramatically; levels of oestrogen also peak at this time and levels of progesterone begin to increase. The high levels of LH stimulate ovulation. During the luteal phase, levels of LH and FSH decrease and the ruptured follicle forms the corpus luteum, which produces progesterone. The corpus luteum degenerates without fertilisation and the decreased levels of progesterone and oestrogen, initiates a new menstrual cycle.
GnRH displays structural conservation across species and all mammals, except the guinea pig, have an identical decapeptide sequence (Gore 2002). In the guinea pig GnRH sequence, tyrosine replaces histidine at position 2 and valine replaces leucine present at position 7 (Gore 2002). The secretion of GnRH from hypothalamic neurons varies throughout human development. Pulsatile secretion of GnRH is noted in early gestation and continues until six months of age in boys and two years in girls. Re-emergence of GnRH secretion does not occur until puberty is approached, when pulsatile secretion of GnRH occurs first during sleep followed by the addition of daytime pulses. Thus the pulse interval and amplitude of GnRH pulses varies throughout development and also during the female menstrual cycle (Neill J.D 2006). LH release stimulated by GnRH is lower in the luteal phase of the menstrual cycle compared with the follicular phase. Pulse interval is 110 minutes in the early follicular, 70 minutes in the mid-follicular and 65 minutes in periovulatory phase. This is more frequent than the luteal phase pulses, which are 100, 200 and 300 minutes in the early, middle and late luteal phase (Gore 2002). Due to other factors such as the action of inhibin, the secretion of FSH, unlike that of LH, does not strictly reflect that of pulsatile GnRH secretion (Clarke, Moore, & Veldhuis 2002; Clarke et al. 1986). The pulsatile secretion of GnRH is necessary for normal reproductive function. Continuous chronic administration of GnRH causes downregulation of the GnRH receptor, resulting in a failure to stimulate pituitary LH and FSH release (Belchetz et al. 1978). Thus GnRH has a pivotal role in regulating reproduction and disordered secretion of GnRH can result in a failure of normal reproductive function.

GnRH neurons have projections to the median eminence where GnRH is released into the hypophyseal-portal capillary circulation (Gore 2002). The onset of puberty and normal reproductive function throughout adulthood is dependent on the pulsatile release of GnRH from the anterior hypothalamic region acting on the anterior pituitary gland to stimulate gonadotrophin release (Belchetz et al. 1978). This “pulse generator” appears to be contained either in the GnRH neuronal network or in the GnRH neurone. In vitro studies have
demonstrated that the hypothalamic neuronal cell line GT1-7 secretes GnRH in a spontaneous pulsatile fashion (Wetsel et al. 1992). In addition GnRH neurones release GnRH in a pulsatile manner without hypothalamic inputs (Funabashi et al. 2000; Terasawa et al. 1999). *In vivo* a number of excitatory and inhibitory factors influence GnRH secretion; these include noradrenaline, neuropeptide Y (NPY), glutamate, gamma-aminobutyric acid (GABA) and endogenous opioids, nitric oxide, cyclic adenosine monophosphate (cAMP), and adenosine triphosphate (ATP) may modify GnRH release without synaptic input (Evans 1999; Terasawa 2001) (Table 1). The precise mechanisms controlling the pulsatile release of GnRH are not fully established. Sex steroid feedback, stress and nutrition also have a role in the regulation of the hypothalamo pituitary gonadal axis. Recently kisspeptin has been identified as a key player in the regulation of GnRH secretion and reproduction.
Table 1. Neurotransmitters acting on Gonadotrophin releasing hormone (GnRH) neurons.

A number of neurotransmitters have either inhibitory, stimulatory or both inhibitory and stimulatory actions on GnRH neurones.

<table>
<thead>
<tr>
<th>Inhibitory</th>
<th>Stimulatory</th>
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<tbody>
<tr>
<td>Acetylcholine</td>
<td>Acetylcholine</td>
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<tr>
<td>Cholecystokinin</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>CRF</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>Dopamine</td>
<td>Delta sleep inducing peptide</td>
</tr>
<tr>
<td>GABA</td>
<td>Dopamine</td>
</tr>
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<td>Opioids</td>
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1.2 Kisspeptin

In 2003 the kisspeptins and their receptor GPR54 (KISS1R) were identified to play a pivotal role in the regulation of reproduction and puberty (de Roux N. et al. 2003; Seminara et al. 2003). In humans the kisspeptins are the neuropeptide products of the KISS1 gene, and are the ligands for the G-protein-coupled receptor KISS1R (GPR54) (Kotani et al. 2001; Lee et al. 1999; Muir et al. 2001; Ohtaki et al. 2001). The KISS1 gene encodes a 145 precursor amino acid peptide which undergoes proteolytic processing to produce shorter peptides. The kisspeptins are named according to amino acid number, kisspeptin-10, 13, 14 and 54; and all share a common C-terminal decapetide necessary for receptor binding (Kotani et al. 2001) (Figure 4). The kisspeptin peptide hormones all have an arginine-phenylalanine residue present at the carboxy terminal (Clements et al. 2001; Kotani et al. 2001), and thus belong to the RF family of peptides.
Figure 4. The amino acid structure of the kisspeptins.

The kisspeptin hormones all have a common carboxy-terminal decapeptide sequence highlighted in grey. This sequence is necessary for biological activity.

Kisspeptin-54

GTLSPPPPESSGSRQQPGLSAPHSRQIPAPQGAVLVQREKDLPNYNWNSFGLRF-NH2

Kisspeptin-14

DLPNYNWNSFGLRF-NH2

Kisspeptin-13

LPNYNWNSFGLRF-NH2

Kisspeptin-10

YNWNSFGLRF-NH2

Kisspeptin 10 is highly conserved between humans and mice, with only one amino acid replacement; tyrosine to phenylalanine (Stafford et al. 2002). The KISS1 gene was initially defined as a metastasis suppressor gene (Lee & Welch 1997) and thus kisspeptin was originally termed metastin. In vivo studies have demonstrated that KiSS-1 suppresses metastasis in human melanoma cells and breast carcinomas (Lee & Welch 1997). Kisspeptin-54 has also been shown to inhibit pulmonary metastasis in a murine melanoma model (Ohtaki et al. 2001). Kisspeptin inhibits the chemotaxis, motility and growth of Chinese Hamster Ovary (CHO) cells with KISS1R, but not that of mock transfectants, thus its actions appear to be mediated via the KISS1R (Ohtaki et al. 2001). The kisspeptin/GPR54 system may have a role in cancer progression (Dhar et al. 2004; Masui et al. 2004; Shirasaki et al. 2001), GPR54 is over-expressed in various forms of malignant tissue compared with normal tissue (Muir et al. 2001; Ohtaki et al. 2001). Plasma kisspeptin levels have been shown to be
elevated in women with placental malignancies such as gestational trophoblastic neoplasia (Dhill et al. 2006). The precise mechanisms by which KiSS-1 expression or GPR54 activation influence metastasis are not yet fully understood.

Data from rodents, sheep, primates and humans has demonstrated KISS1 mRNA to be expressed both centrally in the anteroventral periventricular nucleus (AVPV) or preoptic area and the arcuate or infundibular nucleus; as well as peripheral expression in a number of organs including the placenta, gonads, pancreas and liver (Adachi et al. 2007; Estrada et al. 2006; Gottsch et al. 2004; Kauffman et al. 2007; Kotani et al. 2001; Lee et al. 1999; Muir et al. 2001; Navarro et al. 2004; Ohtaki et al. 2001; Rometo et al. 2007; Shahab et al. 2005; Smith et al. 2007).

The kisspeptin/GPR54 system has an essential role in the reproductive development of humans (de Roux N. et al. 2003; Seminara et al. 2003). Inactivating mutations in the GPR54 gene result in hypogonadotrophic hypogonadism and a failure to undergo puberty in humans and mice (de Roux N. et al. 2003; Funes et al. 2003; Seminara et al. 2003). GPR54 null mice have anatomically normal GnRH neurons with normal levels of hypothalamic GnRH. However, they display phenotypical features of hypogonadotrophic hypogonadism and do not go through puberty, they are phenotypically normal in all other respects (de Roux N. et al. 2003; Funes et al. 2003; Seminara et al. 2003). Administration of GnRH to GPR54 null mice stimulates pituitary gonadotrophin release (Funes et al. 2003; Seminara et al. 2003). In contrast administration of kisspeptin to GPR54 null mice at a dose equivalent to stimulating gonadotrophin release in wild type mice, does not result in gonadotrophin release (Messager et al. 2005). Humans with inactivating mutations in the GPR54 gene have clinical features of hypogonadotrophic hypogonadism (Cerrato et al. 2006; de Roux N. et al. 2003; Lanfranco et al. 2005; Pallais et al. 2006; Seminara et al. 2003; Semple et al. 2005; Tenenbaum-Rakover et al. 2007). They do not display any features associated with abnormal GnRH neuron migration (such as anosmia), and apart from reduced gonadotrophin release, have normal anterior pituitary function (Seminara et al. 2003). Treatment of patients with GPR54
inactivating mutations with GnRH or gonadotrophins has allowed some of these patients to undergo gametogenesis, successful placental function during gestation and lactation (Lanfranco et al. 2005; Pallais et al. 2006; Tenenbaum-Rakover et al. 2007). Thus these findings suggest that inactivating mutations of the GPR54 gene result in disordered GnRH secretion and subsequently hypogonadotrophic hypogonadism, but that the GPR54 may not be vital to maintain pregnancy.

Precocious puberty can be stimulated with administration of kisspeptin to immature prepubertal female rats, as demonstrated by signs of premature vaginal opening, increased uterine weight and stimulation of ovulation (Matsui et al. 2004; Navarro et al. 2004). Kisspeptin can also induce pulsatile LH secretion in juvenile rhesus monkeys, these results appear to be secondary to GnRH stimulation, as the utilisation of GnRH antagonists abolished the effects of kisspeptin (Plant, Ramaswamy, & Dipietro 2006). In humans an activating mutation of GPR54 has been reported to result in central precocious puberty (Teles et al. 2008). The substitution of proline for arginine at amino acid 386 resulted in extended activation of GPR54 receptor with kisspeptin administration (Teles et al. 2008). Three further KISS1R mutations, P74S and H90D (Silveira et al. 2010), and Pro196His (Luan et al. 2007); and a kisspeptin variant Pro110Thr (Luan et al. 2007; Luan et al. 2007); have been identified in idiopathic central precocious puberty. Thus the kisspeptin / GPR54 system has an essential role in pubertal regulation.

1.3 The kisspeptin receptor and kisspeptin signaling

The kisspeptin receptor also known as the GPR54 (abbreviated to KISS1R in humans) is a G protein coupled receptor (GPCR). The KISS1R shares 45% amino acid similarity to the galanin receptor (Lee et al. 1999), despite this homology galanin is not a ligand for the KISS1R.
Binding kisspeptin to its receptor stimulates phospholipase C which in turn activates second messenger systems inositol 1,4,5-trisphosphate and diacylglycerol resulting in increased intracellular calcium (Kotani et al. 2001; Muir et al. 2001; Stafford et al. 2002). The minimum sequence length of kisspeptin required for activation is the ten amino acid sequence at the C terminal (Kotani et al. 2001). Although in vitro studies have demonstrated similar biological activity of the kisspeptins (Kotani et al. 2001); in vivo studies suggest that kisspeptin 54 (KP-54) has a higher potency (Thompson et al. 2006).

In humans there is widespread distribution of the kisspeptin receptor. Highest levels are expressed in the placenta, but the receptor is distributed throughout the body in a number of organs, the central nervous system and peripheral vasculature; these include the cerebral cortex, thalamus, medulla, cerebellum pituitary, pancreas, spinal cord, heart, muscle, kidney, liver, aorta, coronary arteries and umbilical vein (Clements et al. 2001; Kotani et al. 2001; Mead et al. 2007; Muir et al. 2001; Ohtaki et al. 2001).

G protein coupled receptors exhibit receptor desensitisation (Lefkowitz 1998; Ritter & Hall 2009). Acutely desensitisation occurs via loss of signaling to secondary messenger systems due to persistent ligand binding and subsequently receptor internalisation. Degradation rather than recycling of these receptors results in chronic desensitisation (Ferguson 2001). Recently it has been shown that the sequence variant R368P which clinically results in precocious puberty (Teles et al. 2008), reduces the rate of desensitisation of the kisspeptin receptor by decreasing the degradation of the receptor resulting in prolonged responsiveness to kisspeptin (Bianco et al. 2011). Degradation of the kisspeptin receptor appears to be via proteasomes rather than the more common pathway of GPCR degradation which is via lysosomes (Bianco et al. 2011).
1.4 Kisspeptin administration results in stimulation of reproductive hormone release

Kisspeptin administration either via central or peripheral routes stimulates the HPG axis in a number of animal models including rodents, sheep, pigs, primates and humans (Arreguin-Arevalo et al. 2007; Caraty et al. 2007; Dhill et al. 2005; Dhill et al. 2007; Gottsch et al. 2004; Irwig et al. 2004; Lents et al. 2008; Matsui et al. 2004; Messager et al. 2005; Navarro et al. 2004; Plant, Ramaswamy, & Dipietro 2006; Ramaswamy et al. 2007; Seminara et al. 2006; Thompson et al. 2004). Evidence suggests this effect appears to be predominantly mediated via the release of GnRH (Gottsch et al. 2004; Irwig et al. 2004; Matsui et al. 2004; Messager et al. 2005). The use of GnRH antagonists inhibits kisspeptin stimulated increases in gonadotrophins (Gottsch et al. 2004; Irwig et al. 2004; Matsui et al. 2004; Shahab et al. 2005). Intracerebroventricular administration of kisspeptin to sheep results in an increase in cerebrospinal fluid GnRH (Messager et al. 2005). Administration of GnRH to humans and mice with GPR54 mutations results in pituitary release of gonadotrophins suggesting that the kisspeptin/GPR54 system acts upstream of GnRH neurons (Seminara et al. 2003). GPR54 expression has been co-localized to GnRH neurons in rodents (Irwig et al. 2004; Messager et al. 2005) and kisspeptin axons project to GnRH neurons (Clarkson & Herbison 2006). Intracerebroventricular administration of kisspeptin to mice results in increased expression of neuronal markers of activation in GnRH neurons (Irwig et al. 2004). In addition the use of a kisspeptin antagonist at the GPR54 receptor inhibits activation of GnRH neurons and inhibits the release of GnRH and subsequently gonadotrophin release (Roseweir et al. 2009). Kisspeptin is released into the stalk median eminence in a pulsatile fashion with a pattern that demonstrates high concordance with the release of GnRH (Keen et al. 2008).

Sex steroids exert both a negative and positive feedback on GnRH secretion. However the precise mechanisms that regulate these effects are unknown. GnRH neurons do not express oestrogen alpha or androgen receptors (Clarkson & Herbison 2006). In contrast the majority of Kiss1 neurons in the hypothalamus of rodents do express oestrogen alpha receptors.
Thus kisspeptin neurons may relay sex steroid signals to GnRH neurons. In rodents low circulating sex steroids result in an increase in Kiss1 mRNA expression in the arcuate nucleus but a decrease in Kiss1 mRNA expression in the anteroventral periventricular nucleus (Smith et al. 2005a; Smith et al. 2005b). This configuration of kisspeptin expression is reversed with replacement of sex steroids. Thus this rodent model suggests that kisspeptin neurons in the arcuate nucleus may be involved in negative feedback regulation of HPG axis and those in the AVPV with positive feedback regulation of GnRH secretion (Smith et al. 2005a; Smith et al. 2005b). Studies from other species show that gonadectomised animals with subsequently low sex steroid levels exhibit increased Kiss1 expression in the arcuate or infundibular nucleus and increased gonadotrophin levels due to the removal of negative feedback of sex steroids on the HPG axis (Kauffman et al. 2007; Shibata et al. 2007; Smith et al. 2005a; Smith et al. 2005b). Elevated levels of gonadotrophins are not found in gonadectomised GPR54 null mice, even though Kiss1 gene expression is increased in their arcuate nuclei, suggesting that the kisspeptin GPR54 system is pivotal in the negative feedback stimulation of sex steroids on the HPG axis (Dungan et al. 2007).

The use of a kisspeptin antagonist has been demonstrated to inhibit the post castration rise in LH in male mice, which occurs due to removal of negative feedback of sex steroids on the HPG axis. Thus kisspeptin appears to be pivotal in relaying signals between sex steroids and GnRH neuron modulation (Roseweir et al. 2009b).

1.5 Kisspeptin and ovulation

In females positive feedback of oestrogen and progesterone on GnRH secretion triggers the LH surge required for ovulation. Data from rodents suggests that the AVPV is integral in this preovulatory GnRH/LH rise (Herbison 2008; Smith et al. 2006). Rodent models have
demonstrated sexual differentiation in the expression of Kiss1, with an increased expression of Kiss1 mRNA and kisspeptin in the AVPV of females (Adachi et al. 2007; Clarkson & Herbison 2006; Kauffman et al. 2007). In female mice there are an increased number of kisspeptin fibre projections to GnRH neurons compared to male mice (Clarkson & Herbison 2006). Evidence suggests that kisspeptin neurons in the AVPV may be involved in the regulation of ovulation. In female mice the injection of a specific kisspeptin monoclonal antibody into the preoptic area close to GnRH neuron cell bodies, results in elimination of the preovulatory LH surge and inhibits oestrous cyclicity (Kinoshita et al. 2005). Differential hypothalamic expression of KISS-1 is noted during proestrous or experimentally evoked LH surge in female rats; with increased expression of kiss1 and elevated c-fos expression noted in the anteroventral periventricular nucleus in direct contrast to expression in the arcuate nucleus (Smith et al. 2006). In females oestrogen administration results in an increased expression of kiss1 mRNA in the AVPV (Adachi et al. 2007; Smith et al. 2005a; Smith et al. 2006). In addition the majority of kisspeptin neurons in the AVPV express oestrogen receptor alpha, which is proposed to mediate the positive feedback of oestrogen (Glidewell-Kenney et al. 2007; Smith et al. 2005a; Smith et al. 2005b; Wintermantel et al. 2006). Using dual label immunofluorescence techniques Kiss1 neurons appear to have axonal projections to GnRH neurons, with an increase in the number of kisspeptin neuron fibres juxtaposing GnRH neuron cell bodies occurring prior to puberty in the rostral preoptic area of the mouse hypothalamus. However, the absolute numbers of GnRH neurons receiving kisspeptin neuron synaptic connections have not been clarified and inputs to GnRH neuron dendrites has not been established (Clarkson & Herbison 2006). Confirmation of synapse apposition is required with other methods such as electron microscopy. Similar findings have been reported in experiments on female mice, with the identification that kisspeptin neurons in the rostral periventricular area of the third ventricle express oestrogen receptor alpha and progesterone receptors and that these kisspeptin neurons are activated during the GnRH/LH surge (Clarkson et al. 2008).
Importantly, wild type mice which are ovariectomised and supplemented with oestrogen and progesterone are able to generate LH surges, however GPR54 null and Kiss-1 null mice which underwent the same procedure were unable to mount a LH surge and did not display GnRH neuron activation (Clarkson et al. 2008). This highlights the essential role of the kisspeptin/GPR54 system in linking sex steroid signals and GnRH neuron activation required for ovulation. However, a previous study has reported that GPR54 null mice are able to mount an oestrogen induced LH surge, with simultaneous c-Fos induction in GnRH neurons (Dungan et al. 2007). These two studies differed both in the methods used to generate GPR54 knockout mice and in the experimental protocols used to induce and LH surge which may account for the conflicting results (Clarkson et al. 2008; Dungan et al. 2007).

Kisspeptin administration can stimulate ovulation in gonadotrophin primed prepubertal rats with comparable rates to human chorionic gonadotropin (hCG) treatment (Matsui et al. 2004). In addition infusions of kisspeptin can induce timed LH surges and ovulation in progesterone primed cyclical ewes (Caraty et al. 2007); even during anestrous kisspeptin infusions were able to elicit ovulation in acyclic ewes (Caraty et al. 2007). In keeping with these findings in animals, peripheral administration of kisspeptin stimulates gonadotrophin release during each phase of the menstrual cycle in healthy women but its effects were most potent during the preovulatory phase (Dhillo et al. 2007).

1.6 Metabolic regulation of the reproductive axis

Idiopathic hypogonadotrophic hypogonadism, resulting from either a hypothalamic defect in secretion of GnRH or the defective action of GnRH on the pituitary, results in a failure to undergo puberty and infertility. Women with functional hypothalamic amenorrhoea (HA) are hypogonadal or eugonadal, have low oestrogen levels and a lack of menses without any associated organic pathology. As a result these women are infertile and studies indicate that hypothalamic amenorrhoea is a major cause of infertility, accounting for 30% of cases of
amenorrhoea (Reindollar 1986). Hypothalamic amenorrhoea results from a failure of pulsatile GnRH secretion from the hypothalamus and may be triggered by energy deficits associated with weight loss, exercise and eating disorders as well as psychological stress. Recent studies have identified that certain women with rare variants in genes associated with idiopathic hypogonadotrophic hypogonadism may have increased genetic susceptibility for hypothalamic amenorrhoea (Caronia 2011).

Fertility and body nutritional status are closely interwoven, during periods of under nutrition or a lean body weight GnRH production is decreased and thus reproductive ability is reduced or absent (Schneider 2004). By contrast states of obesity can also negatively impact reproductive ability. The hormone leptin is synthesised and secreted by adipocytes in accordance to body energy stores, circulating leptin is proportional to fat stores and falls after weight loss. Leptin has been postulated to relay signals between nutritional status and reproductive functioning. Leptin deficient ob/ob rodent models have hypogonadotrophic hypogonadism with a resultant failure to undergo puberty and infertility (Zhang et al. 1994). Humans with mutations in either the leptin receptor or leptin also display the phenotype of hypogonadotrophic hypogonadism (Farooqi & O'rahilly 2004). LH secretion is suppressed with fasting but these effects are overcome with the administration of leptin in both rodent and primate leptin deficient models (Finn et al. 1998; Nagatani et al. 1998). Leptin administration to juvenile mice stimulates the onset of puberty (Ahima et al. 1996 Chehab, Lim, & Lu 1996). Healthy men and women exposed to calorie restriction have hypoleptinaemia (Chan et al. 2003; Schurgin et al. 2004). Women with hypothalamic amenorrhoea; including those of normal weight, (Miller et al. 1998) have reduced plasma levels of leptin, and treatment with twice daily injections of leptin in these women results in stimulation of gonadotrophin release and ovulation (Chou et al. 2011; Welt et al. 2004).

Leptin does not appear to have a direct action on GnRH neurons (Quennell et al. 2009) and GnRH neurons in the hypothalamus do not express the leptin receptor. Kisspeptin has been proposed as a potential mediator between leptin and GnRH signaling. Forty percent of
kisspeptin neurons in the hypothalamus express the leptin receptor (Smith 2006). In rodent models of leptin deficiency, hypothalamic kiss1 expression is reduced and leptin replacement results in an increase in expression (Smith 2006). Short term fasting leads to decrease in hypothalamic expression of KiSS-1 mRNA and an increase in receptor GPR54 mRNA in prepubertal rats (Castellano et al. 2005). Administration of kisspeptin to rats with delayed puberty due to under nutrition results in restoration of pubertal signs as measured by vaginal opening and gonadotrophin secretion (Castellano et al. 2005). Conversely a recent study has reported that mice lacking the leptin receptor on kisspeptin neurons had normal pubertal development (Donato, Jr. et al. 2011). However, the effects of leptin on kisspeptin neurons may differ in mechanism in specific regions of the hypothalamus, with those kisspeptin neurons in the rodent arcuate nucleus having a direct leptin action on kisspeptin neurons and those in the rostral periventricular area of the third ventricle (RP3V) having an indirect action mediated by currently unknown upstream leptin sensitive neurons (Quennell et al. 2011).

1.7 Potential therapeutic applications of kisspeptin

Kisspeptin may have therapeutic potential in manipulating the human HPG axis via release of GnRH. Various animal models have demonstrated that kisspeptin can stimulate the HPG axis. Two studies in healthy male and female volunteers have also demonstrated its potential in human use with no reported side effects (Dhillo et al. 2005; Dhillo et al. 2007). The first study investigated the effects of intravenous kisspeptin-54 administration to six healthy males in a double blind placebo controlled trial (Dhillo et al. 2005). Compared to placebo, infusion of kisspeptin-54 at a dose of 4 pmol/kg/min resulted in increases of mean plasma LH by 100%, and FSH 18%. The second study of subcutaneous administration of kisspeptin-54 to healthy females demonstrated that kisspeptin can stimulate the HPG axis in females in a dose dependent manner with significant increases in mean plasma LH and FSH; and its effects were most pronounced in the preovulatory phase of the menstrual cycle.
Both these studies confirmed that kisspeptin, similar to animal models, can stimulate reproductive hormone release in humans. They also reported no adverse effects associated with kisspeptin administration. Kisspeptin has been shown to have vasoconstrictor actions *in vitro* (Mead et al. 2007) however *in vivo* studies in humans have not observed any changes in blood pressure or heart rate following kisspeptin administration (Nijher et al. 2010).

Thus kisspeptin has a number of possible therapeutic uses. The stimulation of the HPG axis is required for ovulation and kisspeptin has a potential role in the treatment of infertility and in vitro fertilisation treatment. Kisspeptin stimulates the release of endogenous gonadotrophins, so potentially may offer a more controlled HPG axis stimulation without the risk of excessive stimulation of gonadotrophin release and possible subsequent ovarian hyperstimulation syndrome.

In addition the mode of administration of kisspeptin may determine its therapeutic use; acute administration of kisspeptin stimulates the HPG axis whilst chronic administration of kisspeptin may downregulate the HPG axis (Ramaswamy et al. 2007; Seminara et al. 2006; Thompson et al. 2006). In certain clinical cases such as sex steroid dependent tumours chronic administration of kisspeptin or long acting KISS1R agonists may have a role in achieving medical castration. Exciting studies on the development of kisspeptin antagonists and agonists, will further our understanding of the role of kisspeptin in the regulation of gonadotrophin release and potential therapeutic uses. Specific kisspeptin receptor antagonists have been shown to inhibit the simulation of GnRH neuron firing by kisspeptin (Roseweir et al. 2009). These studies demonstrated that kisspeptin antagonists can inhibit GnRH pulses but do not affect basal GnRH levels in pubertal female rhesus monkeys; additionally there were no effects on basal LH levels but the stimulation of gonadotrophin release by kisspeptin was reduced in uncastrated male mice pretreated with a kisspeptin antagonist. Castration and the subsequent reduction in sex steroids result in removal of negative feedback on the HPG axis and an increase in gonadotrophins; kisspeptin
antagonists were able to inhibit this increase in LH in castrated male mice in a dose
dependent manner. Central administration of a kisspeptin antagonist resulted in a reduced
LH pulse amplitude in ovariectomised ewes. These findings illustrate that kisspeptin is
required for pulsatile GnRH secretion in terms of both frequency and amplitude. The effects
of kisspeptin antagonist on basal LH are exciting as they offer a therapeutic method of
inhibiting pulsatile gonadotrophin release without reducing basal levels. This would offer a
potential new method of contraception (Roseweir et al. 2009).

The kisspeptins share a 10 amino acid c-terminal region required for receptor activation
(Kotani et al. 2001). The development of smaller peptides with GPR54 agonist properties
have demonstrated that the c-terminal five amino acid residues are necessary for GPR54
receptor activation, with the generation of pentapeptide analogues as GPR54 agonists (Niida
et al. 2006; Tomita et al. 2006; Tomita et al. 2007; Tomita et al. 2007). Novel studies of
structure and biological activity of kisspeptin-10 analogues have identified the importance of
combined in vitro and in vivo experiments. Amino acids 6 and 10 appear to be essential for
in vitro kisspeptin-10 GPR54 receptor binding (Gutierrez-Pascual et al. 2009). However in
vivo studies demonstrate that kisspeptin-10 analogues with Ala point substitutions at amino
acid 6 still function as partial agonists with an ability to stimulate gonadotrophin release in
adult male Wistar rats (Gutierrez-Pascual et al. 2009). The development of low molecular
weight oral KiSS-1R agonists and antagonists may offer practical advantages to current
treatments for disorders of reproductive function in humans (Gutierrez-Pascual et al. 2009;
Niida et al. 2006; Orsini et al. 2007; Tomita et al. 2006; Tomita et al. 2008).

1.8 Hypothesis and Aims

Kisspeptin has been identified to be a key regulator in the control of the hypothalamo-
pituitary-gonadal axis. The 145-amino acid precursor of kissepeptin-54 encoded by KISS1 is
also processed to 14, 13 and 10 amino acid sequences (Kotani et al. 2001). Previous work
from our laboratory has shown that kisspeptin-54 stimulates gonadotrophin release in
healthy men and women without any adverse effects (Dhillo et al. 2005, Dhillo et al 2007).
However, no previous studies have examined the effects of kisspeptin in women with infertility due to hypothalamic amenorrhoea. Rodent models of hypothalamic amenorrhoea suggest that there is a decrease in hypothalamic expression of kisspeptin in this condition (Castellano et al. 2005). I hypothesise that women with hypothalamic amenorrhoea, may also have low hypothalamic kisspeptin expression and kisspeptin may restore the functioning of their HPG axis. It is therefore essential that we examine the effects of both acute and chronic kisspeptin administration, as well as different dosing regimes, on women with hypothalamic amenorrhoea.

Animal models suggest that kisspeptin-10 and kisspeptin-54 act similarly to stimulate reproductive hormone release however, kisspeptin-10 is characterised by a shorter half-life. Recently it has been reported that an intravenous bolus of kisspeptin-10 potently stimulates LH secretion while continuous infusion also increases testosterone, LH pulse frequency and amplitude in healthy men (George et al. 2011). In addition kisspeptin-10 appears to reset the GnRH pulsatile clock in men (Chan et al. 2011). The shorter amino acid sequence of kisspeptin-10 makes it simpler and cheaper to synthesize than kisspeptin-54, therefore it may be a more attractive pharmaceutical agent than kisspeptin-54. However, there have been no previous studies on the effects of kisspeptin-10 on women. I hypothesise that kisspeptin-10 will stimulate reproductive hormone release in healthy men and women in a similar fashion to kisspeptin-54.

The aims of this thesis are to investigate the role of kisspeptin in disorders of reproduction specifically investigating:

- The acute and chronic effects of kisspeptin-54 administration on the HPG axis in women with hypothalamic amenorrhoea.
- To compare the effects of different dosing regimens of kisspeptin-54 administration on the HPG axis in women with hypothalamic amenorrhoea.
To investigate the effects of kisspeptin-10 on the HPG axis in healthy men and women.
Chapter 2

The effects of kisspeptin on the hypothalamo pituitary gonadal axis in women with hypothalamic amenorrhoea
2.1 Introduction

Hypothalamic amenorrhoea is a common cause of infertility in women, accounting for 30% of cases of secondary amenorrhoea (Reindollar et al. 1986). In this condition, abnormal GnRH secretion due to deficient GnRH secretion from the hypothalamus or altered pulsatility patterns results in a failure of the synthesis and secretion of gonadotrophins from the pituitary and subsequently a failure in stimulation of the gonads to release sex steroids. This is similar to the phenotype described in hypogonadotrophic hypogonadism. Functional hypothalamic amenorrhoea describes cases where although there is no structural or organic defect identified in the hypothalamic pituitary gonadal axis, patients have amenorrhoea, low or normal serum gonadotrophins and low oestrogen (Reindollar et al. 1986). Functional hypothalamic amenorrhoea may be caused by energy deficient states caused by excessive exercise (Warren 1980), eating disorders (Boyar et al. 1974), weight loss (Frisch 1996; Frisch & McArthur 1974), chronic illness and psychological stress (Giles & Berga 1993) (Meczekalski et al. 2008). There also appears to be a genetic predisposition to hypothalamic amenorrhoea, recently genetic variants in the genes associated with hypogonadotrophic hypogonadism have been identified which may result in increased susceptibility to hypothalamic amenorrhoea in women carrying them (Caronia et al. 2011).

Women with hypothalamic amenorrhoea appear to have leptin deficiency and when given leptin demonstrate restoration of GnRH pulsatility and menstruation (Welt et al. 2004). In a randomised double blinded, placebo controlled 36 week trial of twenty women with hypothalamic amenorrhoea; the administration of subcutaneous injections of human recombinant leptin (metreleptin) to the treatment group resulted in restoration of menstrual cyclicity, with over 50% of cycles being ovulatory (Chou et al. 2011). These authors also note that in addition to biochemical evidence for normalisation of the HPG axis, thyroid and adrenal axis, there were also positive changes in markers of bone formation and resorption.

With regards to treatment, oral contraceptive pills are given to those women who require oestrogen replacement to avoid long term side effects of oestrogen deficiency; these of
course do not restore gonadotrophin release and ovulation and thus do not confer fertility. There is also no evidence as yet that oestrogen and progesterone replacement result in increased bone density (Gordon 2010).

For those women being treating for infertility due to hypothalamic amenorrhoea the options are, clomiphene, GnRH pump therapy or *in vitro* fertilisation (IVF) with gonadotrophin injections. Women with hypothalamic amenorrhoea usually have low serum oestrogen levels (Gordon 2010), thus clomiphene has a poor rate of ovulation induction in these women, as its pharmacologic actions rely on removing oestradiol-mediated negative feedback on pituitary gonadotrophin release and thus increasing circulating gonadotrophin level. Both GnRH pump therapy and exogenous gonadotrophins result in similar rates of ovulation induction in women with hypogonadotrophic amenorrhea (Martin et al. 1993). Although efficacious, both treatment options have individual flaws associated. GnRH pump therapy has practical delivery problems associated with it. Gonadotrophin therapies are associated with risk of ovarian hyperstimulation syndrome (OHSS). Mild forms of ovarian hyperstimulation are common occurring in a third of women having IVF. However, 3-8% of women will have moderate or severe OHSS (HFEA Fertility Facts & Figures 2008). Ovarian hyperstimulation syndrome is a potentially life threatening condition, which can result in massive ovarian enlargement, ovarian torsion, ascites, hydrothorax, liver dysfunction, thromboembolism, electrolyte imbalance, renal failure and acute respiratory distress syndrome and death.

### 2.2 Hypothesis and Aims

Acute central and peripheral administration of kisspeptin results in release of gonadotrophin hormone release in animals. To date human studies on kisspeptin have demonstrated that kisspeptin administration acutely stimulates gonadotrophin release in healthy men and women. Rodent models of hypothalamic amenorrhoea have shown that administration of
kisspeptin-10 restores gonadotrophin secretion and associated pubertal signs (Castellano 2005). Based on these data, I hypothesised that chronic administration of kisspeptin would stimulate reproductive hormone release and restore menstrual cyclicity in human female subjects with HA.

The aim of this study was to determine the acute and chronic effects of subcutaneous administration of kisspeptin-54 compared with placebo saline injections on reproductive hormone release and ovulation in women with hypothalamic amenorrhoea.

2.3 Methods

2.3.1 Subjects

Ethical approval was granted by the Hammersmith and Queen Charlotte’s and Chelsea Hospitals Research Ethics Committee. The study was carried out in accordance with the declaration of Helsinki. All subjects gave full informed written consent prior to study commencement. Recruitment was via advertisements in the local press. After an initial telephone interview, participants were invited for a medical screening. This consisted of a clinical history including a detailed menstrual history, medical examination, electrocardiogram and blood tests. Blood samples were sent to the Imperial College London NHS Trust haematology and biochemistry laboratories and the following assays were conducted: full blood count, renal profile, liver profile, bone profile, thyroid profile, glucose, gonadotrophins, oestradiol, progesterone, androstenedione, dehydroepiandrosterone, testosterone, sex hormone binding globulin, prolactin, 17-hydroxyprogesterone and cortisol. Ten women with functional hypothalamic amenorrhoea were recruited. Hypothalamic amenorrhoea was diagnosed if the following criteria were met: secondary amenorrhoea of at least 6 months duration, body mass index (BMI) of below 25 kg/m² with a stable body weight over the previous 6 months, age between 18–40 years, absence of hormonal contraceptive therapy for one year, absence of systemic disease co-morbidity or active
psychiatric illness; absence of therapeutic or recreational drug use; absence of clinical or biochemical hyperandrogenemia; structurally normal hypothalamus and pituitary region assessed by magnetic resonance imaging; structurally normal female reproductive tract visualised on ultrasound; absence of polycystic ovarian appearances on ultrasound; thyroid function biochemistry and serum prolactin levels within reference range and serum LH:FSH ratio <1.5.

2.3 Kisspeptin 54

Kisspeptin-54 was synthesised by the Advanced Biotechnology Centre, Imperial College London and purified by reverse-phase high performance liquid chromatography (HPLC). Electrospray mass spectroscopy and amino acid analysis confirmed the identity of the peptide. Toxicology testing in animals was conducted prior to administration to volunteers. The Limulus Amoebocyte Lysate assay test for pyrogen (LAL; Associates of Cape Cod, Liverpool, UK) was negative, and the peptide was sterile on culture (Microbiology Department, Hammersmith Hospital, London, UK).

Study 1: Effect of chronic kisspeptin administration on reproductive hormone secretion in women with hypothalamic amenorrhoea

This was a double-blind, placebo-controlled study, which was conducted over eight weeks. Each subject (n = 10) received a subcutaneous (sc) injection of kisspeptin-54 or a control injection of 0.9% saline solution. The dose of kisspeptin-54 used was 6.4 nmol/kg. This dose of kisspeptin-54 was chosen as human studies from our laboratories have identified previously that this dose stimulated the highest gonadotrophin release in healthy women (Dhillo et al. 2007).
Pre study training: each volunteer was trained how to self administer subcutaneous injections into the lower abdominal region. Saline injections were used for training. Subjects were provided with all the necessary equipment to safely self administer their injections and dispose of needles. Subjects were also trained to reconstitute the freeze dried vials containing either control saline or kisspeptin-54 with 0.5ml of 0.9% saline. Each subject was given a specific volume which was calculated according to their weight, to self administer. Subjects were instructed to keep the vials refrigerated.

The study lasted eight weeks in total and this was divided into a 4 week baseline period, followed by a 2 week treatment phase and then a 2 week post treatment observation period. During the 8 week study subjects attended the clinical investigation unit for a review and blood tests twice per week. Measurements of serum gonadotrophin levels and sex hormones and plasma kisspeptin were made at each visit. In addition, pelvic ultrasound scans were performed once a week. These twice weekly measurements allowed calculation of mean levels of LH, FSH and oestradiol during the baseline, treatment and post treatment periods. Compliance was assessed with measurement of plasma kisspeptin immunoreactivity (IR). At each visit pregnancy was excluded using a urine hCG test (Clearview easy HCG, Inverness Medical Innovations Inc. Waltham, MA) (Figure 5).
Figure 5. The 8 week study protocol.

The baseline period was from weeks 1-4, weeks 5 and 6 were the treatment period (shaded black) and the post treatment period was week 7 and 8. During weeks 1 and 2 all subjects were given saline injections to acclimatise them to the study protocol. Each subject was randomised to receive twice daily double blind saline or kisspeptin-54 (KP54) 6.4 nmol/kg injections during weeks 5 and 6. Following the first kisspeptin or saline injection, on day 1 of week 5, a four hour blood sampling study was performed. A second 4 hour study was performed on the last injection day which was on day 7, week 6 of the treatment period. LH pulsatility was assessed at the start of the study and after the treatment period. Ultrasound scans were performed once a week during the 8 week study. Twice weekly blood samples were taken for measurement of LH, FSH, E2 and kisspeptin-IR.
**Baseline period (weeks 1-4)** On the first day of the study subjects had an assessment of their LH pulsatility. This involved the subjects attending the clinical investigation unit for 8 hours, a cannula was inserted into a forearm vein and 2.5 ml of blood was taken every 10 minutes into plain red top serum vacutainer tubes (Becton, Dickinson, Oxford, UK). Subjects remained supine throughout the study. After clotting and centrifugation at 3000rpm for 10 minutes, serum was separated and frozen at -20°C, until measurement of LH and FSH and oestradiol. All the eight hour pulsatility studies were commenced in the morning between 8am and 12pm. Blood pressure and heart rate were monitored every 30 minutes during the pulsatility study.

During weeks 1-2 each volunteer administered twice daily subcutaneous injections of saline. At this stage only volunteers were blinded to the treatment they were receiving as the aim of this baseline period was to allow volunteers to become acclimatised to the study conditions.

**Treatment period (weeks 5-6):** each subject self administered either subcutaneous injections of saline (n=5) or kisspeptin-54 (n=5). Both subjects and investigators were blinded to the treatment assigned. The dose of kisspeptin administered was 6.4 nmol/kg.

On the first day of the treatment period i.e. week 5 day 1 subjects underwent a four hour sampling study post injection of saline or kisspeptin. This was conducted in a dedicated clinical investigation unit. After a clinical review and negative pregnancy urine hCG test, subjects had a cannula inserted into a large forearm vein in the antecubital fossa. Blood was sampled on arrival (t = -30 min) and then at t = 0 min, following which a subcutaneous injection of either saline or kisspeptin-54 6.4 nmol/kg was administered by the investigator. Blood was then sampled at t = 15, 30, 45, 60, 75, 90, 120, 150, 180, 210 and 240 minutes. 3ml of blood was collected into lithium-heparin green top tubes (Becton, Dickinson, Oxford, UK LIP Ltd. Cambridge, UK) containing 5000 kallikrein inhibitor units (0.2ml) aprotinin (Trasylol, Bayer, Newbury, UK). After immediate centrifugation, plasma was rapidly separated and stored at -20°C until measurement of kisspeptin immunoreactivity (IR). An
additional 3ml of blood was collected into plain red top serum vacutainer tubes (Becton, Dickinson, Oxford, UK), these samples were allowed to clot and then centrifuged at 3000 rpm for 10 minutes after which serum was separated and frozen at -20°C until measurement of LH, FSH and oestradiol. Blood pressure and heart rate were measured at every time point. Subjects remained supine throughout the study and were asked if they felt any nausea or other side effects every 30 min. On the last day of the treatment period subjects received only one injection which was administered in the clinical investigation unit. This was followed by a second 4 hour sampling study (Figure 6).

**Figure 6. The four hour study protocol.**

Participants remained in a supine position throughout the four study. A cannula was inserted into a vein in the antecubital fossa on arrival. A randomised subcutaneous injection of either saline or kisspeptin was administered at time zero. Blood samples were taken at regular time intervals from -30 to 240 min.
Post treatment period (weeks 7-8) The day after their final injection (week 7, day 1) subjects had a second assessment of their LH pulsatility over 8 hours. Subjects continued to have twice weekly blood tests taken to measure reproductive hormones and pelvic ultrasounds performed once a week during the two week post treatment period.

Study 2 Effect of chronic kisspeptin administration on GnRH sensitivity in women with hypothalamic amenorrhoea

In order to assess their sensitivity to GnRH pre and post chronic kisspeptin-54 administration; five additional women with hypothalamic amenorrhoea had GnRH stimulation tests performed. These women had a baseline GnRH test conducted seven days prior to commencing twice daily kisspeptin-54 injections for two weeks. These GnRH tests were performed in the clinical investigation unit, women were cannulated and blood was sampled for serum LH, FSH and oestradiol (t=-30), they were then given a 100mcg iv bolus of GnRH (HRF, Intrapharm Ltd, Kent, UK) at time 0. Serial blood samples were taken at times -30, 0, 15, 30, 45, 60, 90, and 120 minutes and serum gonadotrophins and oestradiol were measured. After two weeks of treatment with kisspeptin-54, they underwent a second GnRH test (Figure 7).
Figure 7. The GnRH study protocol.

Five women with hypothalamic amenorrhoea had a GnRH test performed seven days prior to commencing twice daily injections of kisspeptin-54. After this two week treatment period they had a second GnRH test performed. The aim of this study was to assess sensitivity to GnRH pre and post chronic kisspeptin-54 administration.

Measurement of LH, FSH, oestradiol, progesterone, SHBG

LH, FSH, oestradiol and progesterone were measured using automated chemiluminescent microparticle immunoassays (Abbott Diagnostics, Maidenhead, UK). SHBG was measured using a solid-phase two-site chemiluminescent immunometric assay (DPC Immulite, Siemens, Llanberis, UK). Manufacturer stated reference ranges for females were LH (follicular), 2-10 IU/L, (midcycle) 20-60 IU/L (luteal), 4-14; FSH (follicular & luteal) 1.5-8 IU/L, oestradiol (early follicular) less than 300 pmol/l, (luteal) 200-1000 pmol/l; and SHBG 40-80 nmol/l. Interassay coefficients of variation were LH 3.4%, FSH 3.5%, oestradiol 3.4%, progesterone 1.8% and SHBG 5.6%. The limit of detection for oestradiol was 70pmol/l, FSH 0.05 mIU/mL, LH 0.07mIU/ml, progesterone 0.1ng/ml and SHBG 0.1nmol/L.

Kisspeptin radioimmunoassay (RIA)

Antibody GQ2 was raised in a sheep immunised with synthetic human kisspeptin-54 (Bachem UK Ltd.) conjugated to BSA by glutaraldehyde and used at a final dilution of 1:
3,500,000. The antibody cross reacted 100% with human kisspeptin-54, kisspeptin-14, and kisspeptin-10 and less than 0.01% with any other related RFamide peptide including prolactin releasing peptide, RFRP1 (human and rat), RFRP2 (human), RFRP3 (human), neuropeptide FF (human) and neuropeptide AF (human). The $^{125}\text{I}$-kisspeptin-54 label was prepared using the iodogen method (Salacinski et al. 1981) and purified by HPLC. The specific activity of kisspeptin label was 56 Bq/fmol. The assay was performed in duplicate using dilutions of neat plasma in 0.7 ml of 0.06 M phosphate buffer pH 7.2 containing 0.3% BSA and incubated for 3 days at 4ºC. Free and antibody bound label were then separated by charcoal adsorption. The assay detected changes of 2 pmol/l of plasma kisspeptin with a 95% confidence limit. The intra- and inter-assay coefficients of variation were 8.3% and 10.2% respectively.

Ultrasound scans

All the pelvic ultrasound scans were performed in the department of Radiology at Charing Cross Hospital, Imperial College NHS Trust. The scans were transabdominal and performed once a week using Toshiba Apilo XG scanners (Toshiba Medical Systems, Nasu, Japan). Endometrial thickness, mean ovarian volume, mean number of follicles, and the maximum diameter of the largest follicle was measured and recorded at each scan. Ovulation was determined using the following diagnostic criteria: presence of a dominant follicle of diameter 18mm or greater, subsequent collapse of a preovulatory follicle and an associated rise in serum progesterone levels to above 10nmol/l (Pache et al. 1990).

Statistical analysis

All results are presented as mean ± SEM. Statistical comparisons across multiple means were performed using one way ANOVA with the Bonferroni post-hoc correction. Pairs of means were compared using the unpaired two tailed t test. In all cases P<0.05 was
considered to be statistically significant. The GnRH tests and 4 hour studies were analysed using two way ANOVA with the Bonferroni post hoc correction.

Assessment of LH pulses was made using the modified Santen and Bardin method (Adams et al. 1994; Hayes et al. 1999). An LH pulse was defined as consisting of at least two LH points, the first of which was at least 2 IU/l above the preceding nadir and greater than 3 times the CV of the LH assay; and the second point fulfilled one these criteria. LH pulses of three points greater than 1 IU/l above the preceding nadir were also included.
3. Results

Study 1: Effect of chronic kisspeptin administration on reproductive hormone secretion in women with hypothalamic amenorrhoea

The demographics of the women in the kisspeptin and saline control groups is shown in table 2. There were five women with hypothalamic amenorrhea recruited into each group; all the women completed the study. There was no significant difference between the two groups in terms of age, weight, body mass index (BMI), duration of amenorrhea and baseline gonadotrophin and oestradiol levels. Body weight was monitored throughout the study to ensure that all subjects maintained a constant weight. There were no adverse effects reported by subjects or observed in the 4 hour studies post kisspeptin-54 or saline administration.
Table 2. Characteristics of the women in the kisspeptin and saline control groups.

There were five women randomised to either saline or kisspeptin-54 treatment groups. Data is presented as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>Saline control group</th>
<th>Kisspeptin-54 group</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=5</td>
<td>n=5</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>24.8 ± 0.5</td>
<td>26.8 ± 2.4</td>
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<tr>
<td>Weight (kg)</td>
<td>51.8 ± 3.3</td>
<td>54.5 ± 1.1</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>19.0 ± 0.7</td>
<td>19.9 ± 0.4</td>
<td>0.25</td>
</tr>
<tr>
<td>Amenorrhoea duration (months)</td>
<td>22.4 ± 9.9</td>
<td>23.2 ± 12.7</td>
<td>0.96</td>
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<tr>
<td>LH (IU/L)</td>
<td>4.5 ± 1.6</td>
<td>2.6 ± 0.9</td>
<td>0.32</td>
</tr>
<tr>
<td>FSH (IU/L)</td>
<td>6.6 ± 0.7</td>
<td>6.1 ± 1.0</td>
<td>0.69</td>
</tr>
<tr>
<td>Oestradiol (pmol/l)</td>
<td>105 ± 13.9</td>
<td>78 ± 4.9</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Baseline plasma kisspeptin in women with hypothalamic amenorrhoea was <2pmol/L. Following administration of the first dose of kisspeptin-54 there was an acute rise in plasma kisspeptin immunoreactivity (IR) levels which peaked at 45 minutes post injection (Figure 8). There was no increase in plasma kisspeptin IR post injection of saline.
Figure 8. Plasma kisspeptin immunoreactivity (IR) levels over 4 hours post injection of kisspeptin-54 (KP54) 6.4 nmol/kg or saline control on the first day of the study.

Injections were administered at time 0 minutes. Data is shown as mean ± SEM. **p<0.01, ***p<0.001.
A similar response in kisspeptin IR was observed following the last saline or kisspeptin injection on the second 4 hour study. This was conducted on the last day of two weeks of treatment, with either twice daily injections of saline or kisspeptin-54 6.4 nmol/kg (Figure 9).
Figure 9. Plasma kisspeptin immunoreactivity (IR) levels over 4 hours post injection of kisspeptin-54 6.4 nmol/kg or saline control on the last day of two weeks treatment with either twice daily kisspeptin-54 (KP54) 6.4 nmol/kg or saline injections. Injections were administered at 0 minutes. Data is shown as mean ± SEM. *p<0.05, ***p<0.001.
All of the women with HA underwent a 4 week baseline period. During weeks 1 and 2 of this baseline period women received twice daily subcutaneous injections of saline to acclimatise them to the study conditions. Plasma kisspeptin IR levels during this time were <2 pmol/ L. During weeks 5 and 6, which was the treatment period, the women were randomised to receive either twice daily subcutaneous injections of kisspeptin-54 6.4 nmol/kg or saline control. During the treatment period women who received kisspeptin-54 had a significant increase in plasma kisspeptin IR levels (Figure 10A) measured twice weekly. Those who received saline control did not have any increase in plasma kisspeptin IR (Figure 10B).
Figure 10. Weekly plasma kisspeptin IR levels in women with HA randomised to receive subcutaneous injections of (A) kisspeptin-54 6.4 nmol/kg or (B) saline.

Plasma kisspeptin immunoreactivity was significantly elevated during treatment weeks 5 and 6 compared to weeks 1-4, in those randomised to receive kisspeptin-54. Data is shown as mean ± SEM. ***p<0.001
On the first 4 hour study day, which was on the first day of the treatment day, administration of kisspeptin-54 resulted in significant increases in serum gonadotrophins and oestradiol in women with hypothalamic amenorrhoea. There were statistically significant (p<0.001) increases in serum LH levels from baseline from 150 min to 240 minutes post injection of kisspeptin-54. At 240 minutes a maximum increase in LH was noted at 24 ± 3.5 IU/L above baseline. Serum levels of FSH also significantly increased (p<0.001) at 180 to 240 minutes post kisspeptin-54 administration. This increase was not as high as that observed with LH, the maximum rise in FSH was 9.1 ± 2.5 IU/L at 240 minutes. Oestradiol levels showed a late rise, with significant increases only noted after 180 minutes. There were no observed changes in levels of LH, FSH and oestradiol from baseline post injection of saline. (Figure 11).
Figure 11. Changes in serum levels of LH (A), FSH (B) and oestradiol (E2) (C) from baseline after administration of subcutaneous injection of either saline or kisspeptin-54 6.4 nmol/kg (KP54) on the first day of the treatment period.

Injections were administered at 0 min. Data is shown as mean ± SEM *p<0.05, **p<0.01, ***p<0.001
On the last day of the treatment period i.e. after two weeks of twice daily subcutaneous injections of either kisspeptin-54 or saline, saline injection did not result in any change in serum levels of LH, FSH and oestradiol. Although kisspeptin-54 administration did result in a significant increase in serum LH, this was only at 240 minutes post injection. The maximal increase in LH at 240 minutes after an injection of kisspeptin-54 was only 2.48 ± 3.76. There was no significant change in serum FSH and oestradiol levels after kisspeptin-54 administration. All the responses were significantly diminished at each time point compared to those achieved on the first day pre treatment (Figure 12).
Figure 12. Changes in serum levels of LH (A), FSH (B) and oestradiol (E2) (C) from baseline after administration of subcutaneous injection of either saline or kisspeptin-54 6.4 nmol/kg on the last day of the treatment period.

Data is shown as mean ± SEM *p<0.05, **p<0.01, ***p<0.001
A summary of the differences in serum levels of gonadotrophins and oestradiol achieved post injection of kisspeptin-54 on the first and last day of the treatment period is shown in Figure 13. The area under the curve (AUC) represents the total increase in serum LH, FSH and oestradiol achieved in the four hours post injection of kisspeptin-54 6.4 nmol/kg. Significantly lower responses in LH, FSH and oestradiol were achieved post injection of kisspeptin-54 6.4 nmol/kg on day 14 compared to day 1 of the treatment period.
Figure 13. Comparison of the mean area under curve (AUC) increases in LH (A), FSH (B) oestradiol E2 (C) during the four hours post sc injection of kisspeptin-54 6.4 nmol/kg on the first and last injection days on the treatment period.

*p<0.05, **p<0.01, ***p<0.001
The mean levels of serum LH, FSH and oestradiol were calculated for the baseline period (weeks 1-4), the treatment period (weeks 5 & 6) and post treatment (weeks 7 & 8) in both the kisspeptin-54 and saline treated groups (table 3). The mean levels of LH during the baseline period were lower in the kisspeptin-54 treated group compared to those women in the saline study group (p=0.02). During the treatment period the women in the kisspeptin group had small rises in mean levels of LH (p=0.09) and FSH (p=0.02).
Table 3. Comparison of basal serum reproductive hormone levels at baseline, during and after 2 weeks treatment with either saline or kisspeptin injections.

For each subject mean baseline serum levels of LH, FSH and oestradiol were calculated from the blood tests performed during the baseline period of weeks 1-4. Changes in serum levels of LH, FSH and oestradiol levels during the two week treatment period weeks 5-6, and the post treatment period weeks 7-8 were calculated for each subject. Data shown as mean ± SEM.

<table>
<thead>
<tr>
<th>Study group</th>
<th>LH (iU/l)</th>
<th>FSH (iU/l)</th>
<th>Oestradiol (pmol/l)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>Saline</td>
<td>Kisspeptin54</td>
<td>Saline</td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>3.7 ± 0.6</td>
<td>1.8 ± 0.3</td>
<td>109 ± 15</td>
<td>0.02</td>
</tr>
<tr>
<td>Change during 2 week treatment period</td>
<td>-0.8 ± 0.7</td>
<td>+1.2 ± 0.7</td>
<td>+33 ± 48</td>
<td>0.09</td>
</tr>
<tr>
<td>Change during post treatment period</td>
<td>-0.6 ± 0.5</td>
<td>-0.4 ± 0.2</td>
<td>+16 ± 7</td>
<td>0.51</td>
</tr>
<tr>
<td>Change during 2 week treatment period</td>
<td>0.0 ± 0.57</td>
<td>-0.6 ± 0.3</td>
<td>+77 ± 67</td>
<td>0.35</td>
</tr>
<tr>
<td>Change during post treatment period</td>
<td>109 ± 15</td>
<td>91 ± 6</td>
<td>109 ± 15</td>
<td>0.28</td>
</tr>
<tr>
<td>P value</td>
<td>0.02</td>
<td>0.07</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.09</td>
<td>0.02</td>
<td>0.61</td>
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<tr>
<td></td>
<td>0.51</td>
<td>0.35</td>
<td>0.92</td>
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An eight hour LH pulsatility study was performed on day 1 of the baseline period and repeated after two weeks of treatment with either twice daily subcutaneous injections of saline or kisspeptin-54 6.4 nmol/kg. A modified Santen and Bardin method was used to assess LH pulsatility. There were no significant changes in mean LH, number of LH pulses or LH pulse amplitude (Table 4).
Table 4. Comparison of LH pulsatility before and after 2 weeks treatment with saline or kisspeptin-54 6.4 nmol/kg injections in women with HA.

Mean levels of serum LH, number of LH pulses and mean LH pulse amplitude are shown as mean ± SEM.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Study group</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Saline n= 5</td>
<td>Kisspeptin54 n= 5</td>
</tr>
<tr>
<td>Mean LH (iU/l)</td>
<td>Baseline</td>
<td>2.5 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Post-treatment</td>
<td>2.5 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Change</td>
<td>-0.08 ± 0.5</td>
</tr>
<tr>
<td>Number of LH pulses</td>
<td>Baseline</td>
<td>0.8 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Post-treatment</td>
<td>1.6 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Change</td>
<td>+0.8 ± 0.5</td>
</tr>
<tr>
<td>Mean pulse amplitude (iU/l)</td>
<td>Baseline</td>
<td>2.7 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Post-treatment</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Change</td>
<td>-0.4 ± 0.4</td>
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There was no observed change in any of the parameters measured in the transabdominal ultrasound scans. Ovulation was determined using the following diagnostic criteria, presence of a dominant follicle of diameter 18mm or greater, subsequent collapse of a preovulatory follicle and an associated rise in serum progesterone levels to above 10nmol/l (Pache et al. 1990). None of the women ovulated during the eight week study (Table 5).
Table 5. Summary of ultrasound parameters at baseline, during and after 2 weeks treatment with saline or kisspeptin injections in women with HA.

Endometrial thickness, ovarian volume, follicle number and maximum follicle diameter are shown as mean ± SEM. Baseline mean values were calculated from the four scans performed during the baseline period, weeks 1-4. The change in value of each ultrasound parameter was calculated from the two scans during the treatment period (weeks 5-6) and the two scans performed in the post treatment period (weeks 7-8).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Saline</th>
<th>Kisspeptin54</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovulation (number of subjects)</td>
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<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Preovulatory follicle ≥ 18mm (no. of subjects)</td>
<td>1</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Dominant follicle ≥ 11mm (no. of subjects)</td>
<td>1</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Endometrial thickness (mm)</td>
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<tr>
<td>Baseline</td>
<td>3.2 ± 0.4</td>
<td>4.3 ± 0.9</td>
<td>0.29</td>
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<tr>
<td>Change during treatment</td>
<td>+0.4 ± 0.3</td>
<td>-0.5 ± 0.5</td>
<td>0.15</td>
</tr>
<tr>
<td>Change post-treatment</td>
<td>+0.8 ± 0.6</td>
<td>+0.1 ± 0.5</td>
<td>0.36</td>
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<tr>
<td>Ovarian volume (cm³)</td>
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</tr>
<tr>
<td>Baseline</td>
<td>5.2 ± 1.2</td>
<td>5.8 ± 0.6</td>
<td>0.67</td>
</tr>
<tr>
<td>Change during treatment</td>
<td>+0.9 ± 0.4</td>
<td>+1.3 ± 0.8</td>
<td>0.69</td>
</tr>
<tr>
<td>Change post-treatment</td>
<td>+1.2 ± 0.5</td>
<td>+0.7 ± 0.6</td>
<td>0.50</td>
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<tr>
<td>Follicle number per ovary</td>
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<tr>
<td>Baseline</td>
<td>12.4 ± 2.1</td>
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<td>0.79</td>
</tr>
<tr>
<td>Change during treatment</td>
<td>-2.3 ± 1.5</td>
<td>+0.8 ± 1.2</td>
<td>0.13</td>
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<tr>
<td>Change post-treatment</td>
<td>-0.6 ± 1.8</td>
<td>-0.2 ± 1.2</td>
<td>0.86</td>
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<tr>
<td>Maximum follicle diameter (mm)</td>
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<tr>
<td>Baseline</td>
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<td>8.0 ± 0.5</td>
<td>0.11</td>
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<tr>
<td>Change during treatment</td>
<td>+0.5 ± 1.3</td>
<td>+0.1 ± 0.6</td>
<td>0.83</td>
</tr>
<tr>
<td>Change post-treatment</td>
<td>0.0 ± 0.8</td>
<td>+0.2 ± 0.6</td>
<td>0.84</td>
</tr>
</tbody>
</table>
Study 3.2: Effect of chronic kisspeptin administration on GnRH sensitivity in women with hypothalamic amenorrhoea

GnRH tests were performed in order to ascertain if there was an effect of twice daily kisspeptin-54 6.4 nmol/kg subcutaneous injections on the sensitivity of women with HA to GnRH. Although there was a reduction in the gonadotrophin response to GnRH this was not statistically significant different prior to or following two weeks of twice daily kisspeptin-54 injections (p = 0.23) (Figure 14).
Figure 14. Mean gonadotrophin response to GnRH 100 mcg bolus pre (A) and post (B) treatment with twice daily injections of kisspeptin-54 (KP54) 6.4 nmol/kg.

Data is shown as mean ± SEM, n= 5 women with hypothalamic amenorrhoea.
4. Discussion

This is the first study to investigate the effects of kisspeptin-54 in women with infertility due to hypothalamic amenorrhoea. This study has identified that acute administration of kisspeptin to women with HA results in stimulation of reproductive hormones but chronic administration for two weeks of twice daily injections of kisspeptin at a dose of 6.4 nmol/kg results in tachyphylaxis. The second 4 hour study which was conducted after two weeks of treatment with either saline or kisspeptin-54 twice daily showed much lower responses in gonadotrophin and oestradiol stimulation in the kisspeptin group compared to those achieved in the first four study, which was performed on the first day of the treatment period. Tachyphylaxis to the effects of kisspeptin on gonadotrophin release occurred despite retained sensitivity to GnRH, suggesting that the desensitisation was occurring at the level of the KISS1R. Thus as seen in animal models the method of administration of kisspeptin appears to determine its effects on the HPG axis.

The second 4 hour study was conducted using an injection of a vial returned from each subject. To ensure that peptide degradation had not caused the lower gonadotrophin response to kisspeptin at the end of the two week treatment period, plasma kisspeptin levels were measured. Plasma kisspeptin immunoreactivity was similar following the first and last injection of kisspeptin-54. Plasma kisspeptin IR levels were raised for 6 hours post injection of kisspeptin-54. Thus the twice daily administration of kisspeptin54 would have led to prolonged exposure to raised kisspeptin levels. Animal model studies have demonstrated that a continuous exposure to kisspeptin fails to sustain gonadotrophin stimulation. Juvenile agonadal rhesus male monkeys given a 98 hour continuous intravenous infusion of kisspeptin-10 resulted in a stimulation of gonadotrophins for only the initial three hours of the infusion (Seminara et al. 2006). Adult male rats given a three day infusion of kisspeptin-54 also demonstrated gonadotrophin stimulation only in the first 24 hours of the infusion, after
which LH returned to baseline values (Thompson et al. 2006). These effects appear to be secondary to desensitisation of the KISS1R.

A study in adult male rhesus monkeys (Ramaswamy et al. 2007) demonstrated that a 98 hour continuous intravenous infusion of kisspeptin-10 led to reduced responsiveness to GnRH. The monkeys all displayed an acute initial dose related stimulation in gonadotrophin release with a bolus intravenous administration of kisspeptin, however a continuous infusion of 200mcg/hour and a second higher dose infusion of 400 mcg/hour of kisspeptin-10 led to a stimulation in gonadotrophin for only the first 3 hours of the infusion; after which LH fell to baseline levels. In their study Ramaswamy et al. report that there was reduced responsiveness to GnRH which was dose dependent and occurred only with the higher 400mcg/hour infusion (Ramaswamy et al. 2007). We conducted a GnRH study in order to ascertain whether women with hypothalamic amenorrhoea maintained responsiveness to GnRH after two weeks of twice daily kisspeptin-54 6.4 nmol/kg injections. Our results indicate that these women did maintain responsiveness to GnRH, although there was a reduced response in gonadotrophin stimulation, this did not reach statistical significance. Thus the twice daily administration of kisspeptin appears to have led to desensitisation upstream of the GnRH receptor probably at the level of the kisspeptin receptor. A further study examining the effects of kisspeptin administration on GnRH response in healthy women would be interesting. Weight and serum oestradiol levels appear to influence pituitary responsiveness to GnRH (Aono et al. 1975; Knobil 1980). A study of a larger number of women with hypothalamic amenorrhea and healthy women would allow examination of any correlation between BMI and oestradiol levels and the effects of kisspeptin on responsiveness to GnRH.

The effects of twice daily kisspeptin-54 injections at a dose of 6.4 nmol/kg on LH pulsatility were also assessed in this study. We did not demonstrate any statistically significant change in either LH pulse frequency of the mean amplitude of LH increase in women with hypothalamic amenorrhoea. The study by Ramaswamy et al. on the effects of continuous
intravenous infusion of kisspeptin-10 in rhesus male monkeys reported that the high dose of kisspeptin-10 (400mcg/hour) resulted in a reduction in the mean number of LH pulses and LH pulse amplitude (Ramaswamy et al. 2007). These effects were dose related and did not occur with a lower dose (200mcg/hour) of kisspeptin-10. These studies were performed in healthy monkeys with normal GnRH pulsatility. Women with hypothalamic amenorrhoea have abnormal LH pulsatility (Reame et al. 1985) and thus may not be expected to respond in a similar fashion.

Functional hypothalamic amenorrhoea is often a consequence of undernutrition, due either to decreased calorie intake or increased energy output. Rodent models of undernutrition have demonstrated that kisspeptin-10 stimulates higher gonadotrophin responses in undernourished female rats (Castellano et al. 2005). Seventy-two hours of fasting led to a significant decrease in hypothalamic KiSS-1 mRNA levels and increase in GPR54 mRNA expression in male and female prepubertal rats. Intracerebroventricular injections of kisspeptin-10 stimulated higher LH levels in those rats that were fasted compared to those fed ad libitum (Castellano et al. 2005). Comparing the rise in serum gonadotrophins post administration of kisspeptin in women with HA with those achieved in previous studies in healthy women in the follicular phase of the menstrual cycle (Dhillo et al 2007), it was noted that the women with HA appear to more responsive to kisspeptin. They achieved LH levels which were 4 fold greater than those in healthy women. A comparison with the follicular phase of the menstrual cycle was made because similar to the state in hypothalamic amenorrhoea, the follicular phase is characterised by low gonadotrophin and oestradiol levels. The increased expression of hypothalamic kisspeptin receptor GPR54 in rodent models of under-nutrition, (Castellano et al. 2005) may explain the increased potency of kisspeptin stimulation on gonadotrophin release.

This is the first human study showing the effects of acute and chronic administration of kisspeptin-54 to women with hypothalamic amenorrhoea. The results show that after an initial acute stimulation of gonadotrophin release, two weeks therapy of kisspeptin-54 at a
dose of 6.4 nmol/kg led to reduced gonadotrophin response, suggesting desensitisation. The results of this study will have important implications of the use of kisspeptin as a therapy in disorders of reproduction.

Although I did not administer kisspeptin-54 as a continuous infusion, the twice daily injections did lead to raised plasma kisspeptin IR for 6 hours post injection resulting in prolonged exposure to elevated levels of kisspeptin. Animal data has shown that continuous administration of kisspeptin results in desensitisation to its effects on gonadotrophin release (Ramaswamy et al. 2007; Seminara et al. 2006; Thompson et al. 2006). Therefore a different study protocol of kisspeptin-54 administration i.e. a lower dose or reduced frequency of injections may reduce the desensitisation seen in stimulation of gonadotrophin release.
Chapter 3

The effects of chronic twice weekly kisspeptin administration in women with hypothalamic amenorrhea
3.1 Introduction

In Chapter 2 it was demonstrated that a single injection of kisspeptin-54 stimulated gonadotrophin release in women with hypothalamic amenorrhoea. However, women with HA treated for two weeks with twice daily kisspeptin-54 injections had a markedly reduced reproductive hormone response to kisspeptin-54 at the end of the study when compared with the first kisspeptin injection, despite maintaining their responsiveness to GnRH injection. This suggests that desensitisation was occurring at the level of the kisspeptin receptor (KISS1R).

The kisspeptin receptor also known as the GPR54 (abbreviated to KISS1R in humans) is a G protein coupled receptor (GPCR). The binding of kisspeptin to its receptor stimulates phospholipase C which in turn activates second messenger systems inositol 1,4,5-trisphosphate and diacylglycerol resulting in increased intracellular calcium (Kotani et al. 2001; Muir et al. 2001; Stafford et al. 2002). G protein coupled receptors exhibit receptor desensitisation (Lefkowitz 1998; Ritter & Hall 2009). Acutely, desensitisation occurs via loss of signalling to secondary messenger systems due to persistent ligand binding and subsequently receptor internalization. Degradation rather than recycling of these receptors results in chronic desensitisation (Ferguson 2001). Degradation of the kisspeptin receptor appears to be via proteasomes rather than the more common pathway of GPCR degradation which is via lysosomes (Bianco et al. 2011).

Plasma kisspeptin immunoreactivity levels remain elevated for at least four hours following a single subcutaneous injection of kisspeptin-54, therefore twice daily injections of kisspeptin would have led to prolonged exposure to kisspeptin of at least 8 hours a day. Animal models have also shown that chronic administration of kisspeptin-10 to both rats and monkeys results in an acute stimulation of gonadotrophin release followed by a fall in gonadotrophin levels to baseline values when desensitisation occurs.
Acute administration of kisspeptin to women with HA results in a potent stimulation of LH release, but twice daily kisspeptin-54 administration for 2 weeks led to tachyphylaxis (Jayasena et al. 2009f). These data suggest that kisspeptin-54 could potentially be used to downregulate the hypothalamo-pituitary gonadal axis for the treatment of sex hormone dependant tumours and precocious puberty. In addition it is possible that a modified dosing protocol of kisspeptin-54 administration which prevents tachyphylaxis from occurring could be used to stimulate reproductive hormone release long term in women with HA.

3.2 Hypothesis and Aims

I hypothesised that modification of the protocol of repeated kisspeptin administration would reduce the observed desensitisation to its effects in women with HA.

The aims of this study were to determine in women with hypothalamic amenorrhoea

- The time course over which desensitisation to the effects of kisspeptin-54 occurs
- If varying dosing regimens of kisspeptin-54 administration could reduce the desensitisation of the effects of kisspeptin on reproductive hormone release.
- The effects of long-term twice-weekly kisspeptin-54 injections on reproductive hormone release by conducting a randomised, double-blinded placebo-controlled parallel design study over eight weeks in women with hypothalamic amenorrhoea.


3.3 Methods

3.3.1 Subjects

Ethical approval was granted by the Hammersmith and Queen Charlotte’s and Chelsea Hospitals Research Ethics Committee (registration number: 05/Q0406/142). Written informed consent was obtained from all subjects. This study was performed in accordance with the Declaration of Helsinki.

Subjects were recruited through advertisements placed in local newspapers. Responders to adverts were evaluated with a detailed menstrual history, clinical examination and blood tests. Screening blood tests performed were as follows: full blood count; renal profile; liver profile; bone profile; glucose; thyroid profile; LH; FSH; oestradiol; progesterone; androstenedione; dehydroepiandrostenone; testosterone; SHBG; prolactin; 17-hydroxyprogesterone and cortisol. Women were diagnosed with HA and included within the study if they fulfilled the following criteria: body mass index $< 25$kg/m$^2$; stable body weight over the previous 6 months; age between 18 and 40 years; secondary amenorrhea of at least 6 months duration; absence of oral contraceptive pill therapy for at least one year; absence of co-morbidity; absence of active psychiatric illness; stable body weight; absence of therapeutic or recreational drug use; absence of clinical or biochemical signs of hyperandrogenemia; structurally normal hypothalamus and pituitary region assessed by magnetic resonance imaging; structurally normal female reproductive tract visualised on ultrasound; absence of polycystic ovarian appearances on ultrasound; normal thyroid function tests; normal serum prolactin levels; serum LH:FSH ratio $<1.5$. Thirty subjects with HA were recruited to the study.
3.3.2 Study 1: Determination of the time-course of desensitisation to the effects of kisspeptin on gonadotrophin release in women with HA

Five subjects with HA received twice-daily injections of 6.4 nmol/kg of kisspeptin-54. On day 1 (the first injection day), days 2, 3, 4, and day 14 (the last injection day) of the study protocol, each subject underwent a 4 hour sampling study post-injection of kisspeptin.

Protocol for 4 hour blood sampling after injection:

Subjects were admitted to our Clinical Investigation unit and asked to lay supine. Kisspeptin-54 was subcutaneously administered at time 0 minutes by an investigator, and blood was sampled for serum LH, FSH, oestradiol, and SHBG at -30, 0, 15, 30, 45, 60, 75, 90, 120, 150, 180, 210 and 240 minutes (Figure 15).
Figure 15. The four hour study protocol.

Participants remained in a supine position throughout the four studies. A cannula was inserted into a vein in the antecubital fossa on arrival. A randomised subcutaneous injection of either saline or kisspeptin was administered at time zero. Blood samples were taken at regular time intervals from -30 to 240 min as indicated by the arrows.
3.3.3 Study 2: Effects of reduced dose and increased dose-interval on serum reproductive hormone release in women with HA administered kisspeptin injections for 2 weeks

In order to assess the effects of altering kisspeptin-54 dose and dose interval between kisspeptin-54 injections on reproductive hormone release in women with HA, four different 2 week regimes of subcutaneous kisspeptin-54 injections were investigated: 6.4 nmol/kg twice-daily; 1 nmol/kg once-daily; 0.3 nmol/kg once-daily; 6.4 nmol/kg twice-weekly. For each unblinded 2 week pilot study, 5 subjects with HA (see Table 6 for baseline characteristics) attended our clinical investigation unit twice per week. Basal measurements of reproductive hormone were performed twice weekly. On the first (day 1) and last injection day (day 14), the injection of kisspeptin was administered by a study investigator during a 4 hour sampling study.

3.3.4 Study 3: Effects of twice weekly kisspeptin-54 (6.4nmol/kg) administration for 8 weeks on reproductive hormone release in women with HA

Results from Study 2 showed that administration of kisspeptin-54 6.4 nmol/kg twice weekly significantly reduced desensitisation to the effects of kisspeptin-54 on reproductive hormone release. In order to determine if longer term administration of kisspeptin-54 6.4 nmol/kg twice-weekly would continue to stimulate reproductive hormone release a randomised, double blinded, placebo-controlled, parallel design study was performed. Ten subjects with HA (see Table 6 for baseline characteristics) were randomised to receive either saline or kisspeptin 6.4 nmol/kg injections (n=5 per group) twice weekly for 8 weeks.

Each subject attended a hospital investigation unit twice per week (Figure 16). Twice-weekly basal measurements of serum LH, FSH, oestradiol, progesterone and SHBG were taken from subjects throughout the 8 week study protocol between 0800 and 1800h. Trans-abdominal ultrasound scans were performed once a week throughout the 8 week study
protocol. During each scan the following parameters were measured: endometrial thickness in millimetres (mm); mean ovarian volume in cubic centimetres (cm$^3$); number of mean follicles; maximum diameter of largest follicle in each ovary in mm. Ovulation was confirmed by satisfaction of all of the following criteria: visualisation of a dominant follicle (diameter 11mm or greater); enlargement of dominant follicle into a pre-ovulatory follicle (diameter 18mm or greater); subsequent collapse of pre-ovulatory follicle or appearance of internal echoes on ultrasonography; a rise in serum progesterone to over 10nmol/l (Pache et al. 1990).
Figure 16. Protocol to investigate the effects of twice-weekly kisspeptin-54 administration for 8 weeks in women with HA.

Subjects with HA were randomised to receive twice-weekly injections of saline or kisspeptin-54 (KP54) 6.4 nmol/kg for 56 days. Four hour blood sampling was performed immediately following saline or kisspeptin injection on days 1, 14, 28, 42 and 56. Once-weekly ultrasound scans and twice-weekly blood sampling for measurement of LH, FSH, oestradiol (E2) and plasma kisspeptin immunoreactivity (IR) were also performed.
During each visit, a double-blinded subcutaneous injection of either saline or kisspeptin was administered to each subject. Following the first injection of saline or kisspeptin a 4 hour sampling study (Figure 15) was performed in order to investigate the acute effects of the treatment on reproductive hormone release. The 4 hour sampling study was repeated at 2, 4 and 6 weeks, and on the final day of the study protocol after 8 weeks.

During each study visit, urine was tested in order to exclude pregnancy (Clearview easy-HCG, Inverness Medical Innovations Inc. Waltham, MA). Diastolic and systolic blood pressure and heart rate were recorded during 4 hour blood sampling studies performed post-injection of kisspeptin or saline.

3.3.5 Kisspeptin-54

Kisspeptin-54 was synthesised by the Advanced Biotechnology Centre, Imperial College London and purified by reverse-phase high performance liquid chromatography (HPLC). Electrospray mass spectroscopy and amino acid analysis confirmed identity of the peptide as previously described (Dhillo et al. 2005; Dhillo et al. 2007). The peptide was tested for bioactivity and toxicity as previously described (Dhillo et al. 2005). The Limulus amebocyte lysate assay (Associates of Cape Cod, Liverpool, UK) was negative for endotoxin, and the peptide was sterile on culture (Department of Microbiology, Hammersmith Hospital, London). Although kisspeptin-10, -13, -14, and -54 display similar potency in vitro, we used kisspeptin-54 due to its higher in vivo potency than the other kisspeptin fragments (Thompson et al. 2006; Tovar et al. 2006).
3.3.6 Injections of Kisspeptin-54

Vials of freeze-dried saline or kisspeptin were reconstituted in 0.5 ml of 0.9% saline. Then a 0.5 ml insulin syringe was used to inject a weight-adjusted dose of kisspeptin into the lower anterior abdominal region. Depending on the protocol, subjects received kisspeptin doses of 0.3, 1.0 or 6.4 nmol per kg.

Once- or twice-daily injections of kisspeptin: Due to the frequency of these injections, they were self-administered by subjects at home; except during 4 hour blood sampling studies, when injections were administered in our Clinical investigation Unit by an investigator. Prior to commencement of the study protocol, subjects were trained to perform subcutaneous self-injection. A box containing unlabelled vials of freeze-dried saline or kisspeptin-54, alcohol wipes, saline vials, needles and needle disposal bins was given to each subject. Instructions were given to refrigerate vials stored at home.

Twice-weekly injections of kisspeptin or saline: All injections were administered to subjects by study investigators when the subjects attended our clinical investigation unit twice a week.

3.3.7 Collection and processing of blood samples

Blood samples for serum analysis were collected in plain serum Vacutainer tubes (Beckton Dickson, Franklin Lakes, NJ, USA). Clotted samples underwent centrifugation using a Hettich EBA 20 machine (Hettich International, Tuttlingen, Germany) for 10 minutes at 3000rpm. Serum was then separated and stored at -20°C until analysis.
3.3.8 Analytical methods

Serum LH, FSH, oestradiol, and progesterone were measured using automated chemiluminescent immunoassays (Abbott Diagnostics, Maidenhead, UK). SHBG was measured using a solid-phase automated enzyme immunoassay (Immulite; Siemens, Llanberis, UK). Reference ranges for females were as follows: LH (follicular), 2–10 IU/l; LH (midcycle), 20–60 IU/l; LH (luteal), 4–14 IU/l; FSH (follicular and luteal), 1.5–8 IU/l; oestradiol (early follicular), less than 300 pmol/l; oestradiol (midcycle), 400-1500 pmol/l; oestradiol (luteal), 200-1000 pmol/l; and SHBG 40–80 nmol/l. Interassay coefficients of variation were as follows: LH, 3.4%; FSH, 3.5%; oestradiol, 3.4%; progesterone, 1.8%; and SHBG, 5.6%. Limits of detection for each assay were as follows: oestradiol 70pmol/l; FSH 0.05mIU/ml; LH 0.07mIU/ml; progesterone 0.1ng/ml; SHBG 1nmol/l.

3.3.9 Data analysis

Data are presented as mean +/- standard error of mean (SEM). Hormone profiles during 4 hour blood sampling studies were analysed using repeated measures 2-way ANOVA with Bonferroni post hoc correction. Pairs of means were analysed using the unpaired two-tailed t-test. Multiple means were compared using one-way ANOVA with Bonferroni’s Multiple Comparison Test. In all cases, $P < 0.05$ was considered statistically significant.
3.4 Results

Baseline characteristics of age, weight and BMI were similar for all groups of subjects with HA included in this study and are shown in table 6.

Table 6. Comparison of baseline characteristics of women with hypothalamic amenorrhea

Subjects participating in 2 week pilot studies of kisspeptin administration (n=20 in total), and an 8 week study of saline versus kisspeptin administration (n=5 per group). Data shown as mean +/- SEM.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>2 week pilot</th>
<th>8 week study</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kisspeptin54</td>
<td>Saline</td>
<td>Kisspeptin54</td>
</tr>
<tr>
<td>Age (years)</td>
<td>28.1 ± 1.1</td>
<td>27.0 ± 2.6</td>
<td>27.2 ± 2.4</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>52.6 ± 0.7</td>
<td>54.5 ± 3.9</td>
<td>53.4 ± 3.0</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>19.1 ± 0.3</td>
<td>19.7 ± 1.1</td>
<td>19.6 ± 0.7</td>
</tr>
<tr>
<td>Duration of amenorrhea (months)</td>
<td>22.4 ± 9.9</td>
<td>20.4 ± 5.1</td>
<td>28.8 ± 12.0</td>
</tr>
<tr>
<td>Serum LH (iU/l)</td>
<td>2.5 ± 0.6</td>
<td>1.7 ± 0.7</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>Serum FSH (iU/l)</td>
<td>4.8 ± 0.3</td>
<td>3.8 ± 0.9</td>
<td>3.6 ± 0.6</td>
</tr>
<tr>
<td>Serum oestradiol (pmol/l)</td>
<td>133 ± 10.0</td>
<td>87 ± 17.0</td>
<td>151 ± 28.0</td>
</tr>
</tbody>
</table>
Study 1: Time course of desensitisation to the effects of kisspeptin on reproductive hormone release in women with HA

Twice-daily sc administration of 6.4 nmol/kg kisspeptin was associated with a progressive reduction in acute LH responses following injection of kisspeptin, in women with HA (Figure 17A). On the first injection day, the mean AUC LH response during the first 4 hours after kisspeptin injection was 59.3 ± 19.0h.IU/l; however mean AUC LH response during the first 4 hours after kisspeptin injection dropped to 36.0 ± 18.8, 14.9 ± 2.9, 12.9 ± 2.4 and 5.3 ± 1.9h.IU/l on the 2nd, 3rd, 4th and 14th injection days, respectively (Figure 17 A). On the first injection day, the mean AUC FSH response during the first 4 hours after kisspeptin injection was 15.8 ± 3.9h.IU/l; however acute FSH responses following kisspeptin injection were less than 3h.IU/l during the 2nd, 3rd, 4th and 14th injection days (Figure 17 B). Mean oestradiol levels did not significantly change during the study (mean AUC oestradiol response: day 1, 109 ± 119pmol/l; day 14, -65 ± 30pmol/l).
Figure 17. Time course of desensitisation to the effects of kisspeptin-54 (6.4 nmol/kg) on reproductive hormone release administered twice-daily for 2 weeks in women with HA.

Mean area under curve (AUC) LH (A) and FSH responses (B) during the first 4 hours following kisspeptin-54 injections were measured on the 1st, 2nd, 3rd, 4th and 14th days of twice-daily administration of 6.4 nmol/kg sc kisspeptin. Data is shown as mean +/- SEM. * P < 0.05 vs. day 14; *** P < 0.001 vs. day 1.
**Study 2: Effects of reduced dose and increased dose-interval on serum reproductive hormones in women with HA, during a 2-week protocol of kisspeptin injections**

**A: Effect of twice-daily sc injection of kisspeptin-54 (6.4 nmol/kg) on reproductive hormone release.**

Twice-daily sc injection of 6.4 nmol/kg kisspeptin-54 to women with HA led to a potent stimulation of gonadotrophin release on the first injection day, with the maximal increase observed 240 minutes after injection (mean maximal increase following injection: LH: 11.2 ± 4.6 IU/l; FSH: 7.2 ± 2.1 IU/l). However on the last (14th) injection day, reproductive hormone responses were significantly reduced when compared with responses on the first injection day (mean maximal increase following injection on the 14th injection day: LH: 1.0 ± 0.5 IU/l, P<0.05 when compared with 1st injection day; FSH: 0.2 ± 0.3 IU/l, P<0.001 when compared with 1st injection day) (Figure 18 A, B). Furthermore the oestradiol response was non-significantly lower on the 14th injection day when compared with the first injection day (mean maximal oestradiol increase following injection: first injection day, 48.2 ± 29.7 pmol/l; 14th injection day, -12.7 ± 7.0 pmol/l, P=0.06) (Figure 18 C).
Figure 18. Serum reproductive hormone levels following twice-daily regimens of kisspeptin-54 6.4 nmol/kg injection in 5 women with HA.

Changes in serum LH (A), FSH (B) and oestradiol E2 (C); over 4 hours after sc kisspeptin-54 (KP54) on the first day and 14th day following twice daily sc injection of 6.4 nmol/kg KP54.
B: Effect of once-daily sc injection of kisspeptin-54 (1 nmol/kg) on reproductive hormone release.

We then investigated if protocols using lower cumulative doses of kisspeptin treatment would reduce the observed desensitisation in subjects with HA. First, we examined the effects of a protocol of once-daily 1 nmol/kg kisspeptin-54 injections in 5 further subjects with HA. On the first injection day, the maximal LH and FSH responses were $3.7 \pm 0.9$ pmol/l and $1.9 \pm 0.3$ pmol/l, respectively (Figure 19 A, B). However on the 14th injection day, LH and FSH responses were significantly reduced when compared with responses on the first injection day (mean maximal increase following injection on the 14th injection day: LH: $1.5 \pm 0.2$ IU/l, $P<0.05$ when compared with 1st injection day; FSH: $0.3 \pm 0.2$ IU/l, $P<0.005$ when compared with 1st injection day) (Figure 19 A, B). The oestradiol response was non-significantly lower on the 14th injection day when compared with the first injection day (mean maximal oestradiol increase following injection: first injection day, $48.6 \pm 29.3$ pmol/l; 14th injection day, $0.8 \pm 13$ pmol/l, $P=0.11$) (Figure 19 C).
Figure 19. Serum reproductive hormone levels following once daily dosing regimens of kisspeptin-54 1 nmol/kg injection in 5 women with HA.

Changes in serum LH, FSH, and oestradiol E2 over 4 hours following once daily sc injection of 1 nmol/kg of KP54. Data is shown as mean +/- SEM. * P < 0.05; *** P < 0.001
We then performed a 2 week study of once-daily kisspeptin-54 injections at a lower dose of 0.3 nmol/kg in order to determine if desensitisation would still occur with further reduced exogenous kisspeptin exposure. On the first injection day, following kisspeptin-54 (0.3 nmol/kg) injection the peak LH was only moderately increased, and non-significantly reduced after 14 days of kisspeptin treatment (mean maximal LH increase following injection: first injection day, 5.3 ± 1.8 IU/l; 14th injection day, 1.2 ± 0.4 IU/l, P=0.10). FSH responses followed a similar pattern to LH response (mean maximal FSH increase following injection: first injection day, 0.8 ± 0.6 IU/l; 14th injection day, -0.6 ± 0.5 IU/l, P=0.20) (Figure 20 A, B). The peak oestradiol response was below 20 pmol/l on the first injection day, and non significantly lower on the 14th injection day (Figure 20 C).
Figure 20. Serum reproductive hormone levels following once daily dosing regimens of kisspeptin-54 0.3 nmol/kg injection in 5 women with HA.

Changes in serum LH (A), FSH (B), and oestradiol (C); over 4 hours following once daily sc injection of 0.3 nmol/kg of KP54.
**D: Effect of twice-weekly sc injection of kisspeptin-54 (6.4 nmol/kg) on reproductive hormone release.**

I had observed that 1.0 nmol/kg once-daily kisspeptin-54 sc administration led to significant desensitisation of gonadotrophin responses after 2 weeks of administration. Although I did not observe significant desensitisation with 0.3 nmol/kg once-daily kisspeptin-54 sc administration, the absolute rises in reproductive hormone levels at this dose were modest. I therefore studied the effects of further reducing the dose-interval of sc kisspeptin-54 administration to twice-weekly, at a dose of 6.4 nmol/kg per injection. On the 14th injection day, mean gonadotrophin responses following injection were non-significantly lower on the 14th injection day when compared with the first injection day (mean maximal increase in IU/l following injection: LH: 18.8 ± 6.6 vs. 11.5 ± 4.0, P=0.08; FSH: 5.8 ± 2.0 vs. 4.1 ± 1.1, P=0.14) (Figure 21 A, B). The mean maximal increase in serum oestradiol following kisspeptin injection was similar on the 1st and 14th injection days (1st day: 44.8 ± 15.7 pmol/l vs. 14th day: 27.5 ± 15.5 pmol/l; P=0.47) (Figure 21 C).
Figure 21. Serum reproductive hormone levels following twice weekly daily dosing regimens of kisspeptin-54 6.4 nmol/kg injection in 5 women with HA.

Changes in serum LH (A), FSH (B) and oestradiol E2 (C); over 4 hours following twice weekly sc injection of 6.4 nmol/kg of KP54.
A summary of the effects of sc kisspeptin-54 on the first and last day of each of these different 2 week dosing regimens of kisspeptin-54 is shown as a mean area under the curve for LH, FSH and oestradiol in Figures 22 A-C.
Figure 22. Serum reproductive hormone levels following different 2 week dosing regimens of kisspeptin-54 injection in women with HA.

The effects of sc injection of KP54 on the first day and the 14th day of each of the different 2 week dosing regimens of KP54 is shown as a mean area under the curve for LH, FSH and oestradiol in (A-C). Data is shown as mean +/- SEM. * P < 0.05; ** P < 0.01.
6.4nmol/kg twice-daily
0.3nmol/kg once-daily
1nmol/kg once-daily
6.4nmol/kg twice-weekly

AUC E2 increase (h.pmol/L)
Study 3: Effects of twice-weekly kisspeptin-54 administration for 8 weeks on reproductive hormone levels in women with HA.

Baseline age, weight and BMI were not significantly different between kisspeptin and saline study groups (Table 6). Subjects reported no side effects following injection of kisspeptin or saline. No significant acute changes in heart rate, systolic and diastolic blood pressure were observed following kisspeptin administration when compared with saline control.

Saline had no significant effects on reproductive hormone release at any time during the 8 week protocol of twice-weekly injections (Figure 23 A-C).
Figure 23. Serum reproductive hormone levels following twice-weekly saline injection for 8 weeks in women with HA.

Changes in serum LH (A), FSH (B) and oestradiol E2 (C) after bolus sc injection of saline (n=5) on first day, and after 2, 4, 6 and 8 weeks of administration. Data is shown as mean +/- SEM. ** P < 0.01 vs. baseline; *** P < 0.001 vs. baseline.
As expected, kisspeptin-54 stimulated reproductive hormone release following administration on the first injection day. After 2 weeks of twice-weekly injections, LH responses following kisspeptin injection were significantly lower than LH responses following injection on the first injection day (mean maximal LH increase (IU/l): baseline, 21.5 ± 10.7; 2 weeks, 10.0 ± 4.3; P<0.001) (Figure 24 A). However, no further significant reductions in LH following injection of kisspeptin were observed at 4 weeks (mean maximal LH increase: 9.0 ± 4.1 IU/l; P>0.05 vs. response at 2 weeks), 6 weeks (mean maximal LH increase: 8.9 ± 3.5 IU/l; P>0.05 vs. response at 2 weeks) or 8 weeks (mean maximal LH increase: 7.9 ± 4.5 IU/l; P>0.05 vs. response at 2 weeks) (Figure 24 A). FSH responses following kisspeptin injection changed in a similar manner to LH responses over the 8 week protocol (mean maximal increase in serum FSH following kisspeptin injection (IU/l): baseline, 6.4 ± 3.2; 2 weeks, 2.7 ± 0.7, P<0.001 vs. baseline response; 4 weeks, 2.6 ± 0.7, P>0.05 vs. response at 2 weeks; 6 weeks, 2.4 ± 0.8, P>0.05 vs. response at 2 weeks; 8 weeks, 2.7 ± 0.8, P>0.05 vs. response at 2 weeks) (Figure 24 B). Oestradiol responses following injection of kisspeptin were similar throughout the 8 week protocol of twice-weekly injections (mean maximal increase in serum oestradiol following kisspeptin injection (pmol/l): baseline, 44.4 ± 19.9; 2 weeks, 39.2 ± 14.8; 4 weeks, 46.2 ± 25.8; 6 weeks, 60.8 ± 17.2; 8 weeks, 20.0 ± 7.5) (Figure 24 C).
Figure 24. Serum reproductive hormone levels following twice weekly kisspeptin-54 injection for 8 weeks in women with HA.

Changes in serum LH (A), FSH (B) and oestradiol E2 (C) after bolus sc injection of 6.4 nmol/kg kisspeptin-54 (n=5) on first day, and at after 2, 4, 6 and 8 weeks of administration. Injections were administered at 0 min. Data is shown as mean +/- SEM. ** P < 0.01, 2 weeks vs. day. *** P < 0.001, 2 weeks vs. baseline.
No significant changes in the twice-weekly basal reproductive hormone measurements were observed at any stage during the 8 week protocol, between subjects with HA receiving saline and kisspeptin injections (Table 7). No significant changes in follicle number, maximum follicle size, ovarian volume, or endometrial thickness were observed at any stage of the 8 weeks protocol between subjects with HA who received kisspeptin and saline injections (Table 8). Although dominant follicles were observed in some subjects of both treatment groups, no preovulatory follicles were observed in any subject during the study. One subject receiving saline treatment developed a preovulatory LH surge after 8 weeks of saline treatment; however no subjects ovulated during the study.
Table 7. Comparison of basal serum reproductive hormone levels at baseline, during and 8 weeks of treatment with either saline or kisspeptin injections.

Mean ± SEM basal serum hormone levels are provided for subject groups randomised to receive saline (n=5) or kisspeptin-54 (n=5). For each subject, baseline serum LH, FSH and oestradiol levels were measured at baseline, and calculated as the mean levels from separate blood tests performed during baseline, week 2, weeks 3-4 and weeks 5-8 of the study protocol.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Study group</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>Kisspeptin 54</td>
</tr>
<tr>
<td>LH (iU/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.5 ± 0.6</td>
<td>1.8 ± 0.7</td>
</tr>
<tr>
<td>Week 2</td>
<td>1.9 ± 0.5</td>
<td>1.7 ± 0.6</td>
</tr>
<tr>
<td>Weeks 3-4</td>
<td>1.9 ± 0.5</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>Weeks 5-8</td>
<td>3.7 ± 2.7</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>FSH (iU/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>3.7 ± 0.6</td>
<td>3.4 ± 0.5</td>
</tr>
<tr>
<td>Week 2</td>
<td>2.8 ± 0.8</td>
<td>4.5 ± 0.9</td>
</tr>
<tr>
<td>Weeks 3-4</td>
<td>3.1 ± 1.0</td>
<td>3.1 ± 0.9</td>
</tr>
<tr>
<td>Weeks 5-8</td>
<td>3.2 ± 0.8</td>
<td>4.3 ± 0.7</td>
</tr>
<tr>
<td>Oestradiol (pmol/l)</td>
<td>Baseline</td>
<td>97 ± 13.0</td>
</tr>
<tr>
<td></td>
<td>Week 2</td>
<td>91 ± 10.0</td>
</tr>
<tr>
<td></td>
<td>Weeks 3-4</td>
<td>113 ± 33.0</td>
</tr>
<tr>
<td></td>
<td>Weeks 5-8</td>
<td>123 ± 37.0</td>
</tr>
</tbody>
</table>
Table 8. Summary of ultrasound parameters at baseline and throughout the 8 week treatment with saline or kisspeptin injections, in women with HA.

Subjects randomised to receive saline (n=5) or kisspeptin-54 (n=5). Baseline mean values were calculated from results of four separate scans performed during weeks 1-4 of the study protocol. The change in value of each ultrasound parameter during weeks 5-6 (during treatment) or 7-8 (post-treatment), was based upon results of two separate scans performed during the respective 2 week period. Data shown as mean ± SEM.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Study Group</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>kisspeptin-54</td>
</tr>
<tr>
<td>Ovulation (number of subjects)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Preovulatory follicle ≥ 18mm (no. subjects)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dominant follicle ≥ 11mm (no. subjects)</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Endometrial thickness (mm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weeks 1-2</td>
<td>2.8 ± 0.1</td>
<td>3.2 ± 0.5</td>
</tr>
<tr>
<td>Weeks 3-4</td>
<td>3.2 ± 0.4</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>Weeks 5-6</td>
<td>2.9 ± 0.3</td>
<td>4.0 ± 0.5</td>
</tr>
<tr>
<td>Weeks 7-8</td>
<td>3.2 ± 0.7</td>
<td>4.0 ± 0.5</td>
</tr>
<tr>
<td>Ovarian volume (cm³)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weeks 1-2</td>
<td>5.7 ± 1.8</td>
<td>6.7 ± 2.1</td>
</tr>
<tr>
<td>Weeks 3-4</td>
<td>5.2 ± 1.5</td>
<td>5.3 ± 1.0</td>
</tr>
<tr>
<td>Weeks 5-6</td>
<td>5.4 ± 1.3</td>
<td>5.2 ± 0.6</td>
</tr>
<tr>
<td>Weeks 7-8</td>
<td>6.1 ± 1.9</td>
<td>5.5 ± 0.7</td>
</tr>
<tr>
<td>Follicle number per ovary</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weeks 1-2</td>
<td>12 ± 4.0</td>
<td>13 ± 3.0</td>
</tr>
<tr>
<td>Weeks 3-4</td>
<td>11 ± 3.0</td>
<td>15 ± 3.0</td>
</tr>
<tr>
<td>Weeks 5-6</td>
<td>11 ± 3.0</td>
<td>16 ± 3.0</td>
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<tr>
<td>Weeks 7-8</td>
<td>9.0 ± 4.0</td>
<td>13 ± 4.0</td>
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<tr>
<td>Maximum follicle diameter (mm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weeks 1-2</td>
<td>5.0 ± 1.0</td>
<td>7.0 ± 0.9</td>
</tr>
<tr>
<td>Weeks 3-4</td>
<td>6.5 ± 1.5</td>
<td>7.8 ± 1.6</td>
</tr>
<tr>
<td>Weeks 5-6</td>
<td>6.0 ± 1.1</td>
<td>7.3 ± 0.9</td>
</tr>
<tr>
<td>Weeks 7-8</td>
<td>6.3 ± 0.8</td>
<td>7.3 ± 1.2</td>
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5. Discussion

Intact kisspeptin signalling is pivotal in regulating the HPG axis and essential for reproductive maturation in humans (de Roux N. et al. 2003; Seminara et al. 2003). Kisspeptin receptor antagonists disrupt the activity of the hypothalamic-pituitary-gonadal (HPG) axis in adult monkeys, sheep and rodents (Roseweir et al. 2009). Hence there is a need to evaluate the therapeutic potential of kisspeptin. Short-term clinical studies suggest that kisspeptin safely stimulates reproductive hormone release; however chronic administration has until now been shown to cause profound tachyphylaxis in humans (Jayasena et al. 2009).

The paradoxical effects of kisspeptin on reproductive hormone release has been demonstrated in monkeys; intermittent, hourly intracerebroventricular injections of kisspeptin-10 to monkeys stimulates pulsatile LH release (Plant, Ramaswamy, & Dipietro 2006), whereas continuous icv infusion of kisspeptin-10 to monkeys leads to desensitisation of gonadotrophin release within 3 hours of commencement of the infusion (Ramaswamy et al. 2007; Seminara et al. 2006). The phenomenon of desensitisation has also been demonstrated in rodents (Ramaswamy et al. 2007; Roa et al. 2008; Thompson et al. 2006). I have presented detailed data of the time-course of desensitisation to the effects of kisspeptin-54 on reproductive hormone release in women with HA. My data suggests that desensitisation of LH response to twice-daily kisspeptin-54 sc administration in women with HA, occurs gradually over the 14 day injection period. LH responses on the 4th injection day were significantly higher than on the 14th injection day. By contrast, FSH responses dropped to less than 3 h.IU/l after just 24 hours of twice-daily kisspeptin administration. Interestingly, Roa et al. showed that constant icv infusion of kisspeptin-10 to adult female cycling rats led to desensitisation of LH response by day 3, but desensitisation of the FSH response to kisspeptin took longer (Roa et al. 2008). The reason for this discrepancy between human and rodent FSH responses to kisspeptin is unclear. However, it is possible that icv and
peripherally administered kisspeptin have differential effects on gonadotrophin release. Furthermore, we cannot exclude that humans and rodents respond differently to kisspeptin, or indeed that different kisspeptin peptides (kisspeptin-54 used in our studies compared to kisspeptin-10 used by Roa et al. (Roa et al.2008) have differential effects on FSH release.

In order to reduce the effect of desensitisation associated with repeated administration of kisspeptin (6.4 nmol/kg twice daily); I lowered the dose of sc kisspeptin injection by studying the effects of once-daily administration. Interestingly, once-daily 1 nmol/kg (total dose 14 nmol/kg over 2 weeks) of kisspeptin administration still led to significant desensitisation of gonadotrophin responses in women with HA. I did not observe significant desensitisation with 0.3 nmol/kg (total dose 4.2 nmol/kg over 2 weeks) of kisspeptin. However, even on the first injection day, the stimulation of reproductive hormone release following 1.0 and 0.3 nmol/kg sc kisspeptin injection was only moderate due to the reduced dose of kisspeptin used. Furthermore, on the 14th injection day residual LH and FSH responses following once-daily kisspeptin injection (0.3 or 1.0 nmol/kg) and twice-daily kisspeptin injection (6.4 nmol.kg) were similar. Plasma kisspeptin immunoreactivity following a single subcutaneous injection of kisspeptin-54 persists for up to 6 hours in women with HA (Jayasena et al. 2009). My results therefore suggest that once-daily kisspeptin administration of kisspeptin leads to tachyphylaxis, even at low doses. This data suggests that either once-daily or twice-daily protocol of sc kisspeptin administration leads to tachyphylaxis. This has important implications for the future development of any kisspeptin-based endocrine therapy used to perform medical castration in patients with sex hormone-sensitive tumours.

In view of the tachyphylaxis observed with once-daily administration of kisspeptin, I determined if lengthening the dose interval to twice-weekly would allow recovery from desensitisation to kisspeptin. The dose of 6.4 nmol/kg (total dose 32 nmol/kg over 2 weeks) kisspeptin was chosen, since acute injection of kisspeptin-54 at this dose results in adequate stimulation of gonadotrophin levels in women with HA (Jayasena et al. 2009). I performed a 2 week pilot study which suggested that gonadotrophin responses were only
partially diminished after twice-weekly 6.4 nmol/kg kisspeptin administration. This suggests that partial recovery of desensitisation of the kisspeptin receptor may have occurred during the interval between each injection of kisspeptin.

In order to further assess the regime of twice-weekly 6.4 nmol/kg kisspeptin sc injection, I performed a longer 8 week study of its administration. As observed during the pilot study, this regime of kisspeptin administration still lead to partial desensitisation in gonadotrophin responses during the first 2 weeks of administration. However gonadotrophin responses to kisspeptin injection did not significantly diminish further beyond this initial 2 week period. My results therefore suggest that subjects with HA remained partially responsive to kisspeptin injection during the 8 week study period, and that some recovery from desensitisation had occurred between injections. Interestingly oestradiol responses to kisspeptin remained remarkably constant across the entire 8 week period, despite the initial reduction in pituitary responsiveness observed during the first 2 weeks of kisspeptin administration. It is possible that the initial stimulation of oestradiol secretion caused by kisspeptin administration at the beginning of the study, acted to further sensitise the ovaries to further gonadotrophin stimulation. On the other hand, it cannot be excluded that the stimulatory actions of kisspeptin on the ovaries are in part explained by a direct stimulatory action. Kisspeptin receptors are expressed on human ovaries (Gaytan et al. 2009), but no direct stimulatory action of kisspeptin on ovarian tissue has been reported.

During the 8 week study of kisspeptin administration, I did not observe any significant changes in follicle growth when compared with saline administration. This might have reflected that FSH responses were lower than LH responses following kisspeptin administration, which is in agreement with previous data (Dhillo et al. 2005; Dhillo et al. 2007; Jayasena et al. 2009). Furthermore as discussed, I observed that FSH responses desensitised more rapidly following kisspeptin injection than LH responses. Thus, inadequate amplitude and duration of FSH stimulation might have accounted for the lack of detectable ovarian follicular growth in this study.
In summary, I have determined the time-course of desensitisation of LH and FSH responses during twice-daily administration of kisspeptin-54 in women with HA. Once or twice-daily administration of kisspeptin are associated with desensitisation, which may be utilised therapeutically in the treatment of hormone-sensitive tumours. I have also conducted the first long-term clinical study of kisspeptin administration. A single injection of kisspeptin still robustly elicits reproductive hormone release in women with HA, even after 2 months of twice-weekly kisspeptin-54 administration. These findings have important therapeutic implications; kisspeptin-54 may be a novel therapeutic tool for chronically inducing reproductive hormone release in HA and other conditions associated with hypogonadotrophic hypogonadism.
Chapter 4

The Effects of Kisspeptin-10 on Reproductive Hormone Release Show Sexual Dimorphism in Humans
4.1 Introduction

The KISS1 gene encodes a 145-amino acid precursor protein, this undergoes proteolytic processing to generate 54, 14, 13 and 10 amino acid sequences (Kotani et al. 2001). These shorter peptides are named according to their number of constituent amino acids; they all share a common C terminal decapeptide sequence, which is required for \textit{in vitro} biological activity. There have been a number of studies examining the effects of kisspeptin-10 in animals. These studies have demonstrated that central or peripheral administration of kisspeptin-10 to rodents, sheep, monkeys, hamsters, pigs and cows (Irwig et al. 2004; Navarro et al. 2004; Gottsch et al. 2004; Messager et al. 2005; Caraty et al. 2007; Plant et al. 2006; Ramaswamy et al. 2007; Shahab et al. 2005; (Lents et al. 2008); (Kadokawa et al. 2008), stimulates gonadotrophin release. The stimulatory effects of kisspeptin-10 on gonadotrophin release are inhibited by the central administration of a GnRH antagonist.

Studies examining the effects of kisspeptin-54 in humans have identified that kisspeptin-54 stimulates gonadotrophin release in both healthy men and women (Dhillo et al. 2005; Dhillo et al. 2007). In women these effects are most pronounced in the preovulatory phase of the menstrual cycle (Dhillo et al. 2007). Kisspeptin-54 has also been shown to stimulate gonadotrophin release in women with a model of infertility due to hypothalamic amenorrhoea (Jayasena et al. 2009; Jayasena et al. 2010). Human male studies have demonstrated that an intravenous bolus injection of kisspeptin-10 potently stimulates LH secretion while a continuous infusion of kisspeptin-10 also increases testosterone, LH pulse frequency and amplitude (George et al. 2011). Administration of kisspeptin-10 appears to reset the GnRH pulsatile clock, by inducing an immediate LH pulse, regardless of the timing of the previous pulse and these pulses were on average of greater amplitude than endogenous pulses (Chan et al. 2011). The effects of kisspeptin-10 administration on women are not known.
Compared to kisspeptin-54, kisspeptin-10 has a shorter half-life and faster onset of action after intravenous administration in rodents (Mikkelsen et al. 2009). As kisspeptin-10 is simpler and cheaper to manufacture due to its shorter amino acid sequence, future kisspeptin based reproductive therapies may be based upon kisspeptin-10 rather than kisspeptin-54. It is therefore therapeutically important to determine whether kisspeptin-10 can stimulate reproductive hormone release in healthy men and women.

4.2 Hypothesis and Aim

Animal models suggest that kisspeptin-10 and kisspeptin-54 act similarly to stimulate reproductive hormone release however, kisspeptin-10 is characterised by a shorter half-life. Recently it has been reported that administration of kisspeptin-10 potently stimulates LH pulse frequency and amplitude in healthy men (George et al. 2011). In addition kisspeptin-10 appears to reset the GnRH pulsatile clock in men (Chan et al. 2011). There have been no previous studies on the effects of kisspeptin-10 on women. I hypothesise that kisspeptin-10 will stimulate reproductive hormone release in healthy men and women in a similar fashion as kisspeptin-54.

This study aimed to determine the effects of kisspeptin-10 administration on reproductive hormone release in healthy men and, for the first time, in healthy women.

4.3 Methods

4.3.1 Subjects

This study was conducted with Ethics Committee approval (reference 08/H0707/95) in accordance with The Declaration of Helsinki. Recruitment was via advertisements in the
local press. Full written informed consent was obtained from all subjects, 25 healthy female volunteers and 11 male volunteers were recruited after medical screening (Table 9). Recruitment involved a clinical history, clinical examination, electrocardiogram, and blood tests (full blood count, renal profile, liver and bone profile, thyroid profile, random glucose, prolactin, gonadotrophins and sex hormone profile). Men and women were included in the study if they fulfilled the following criteria; age 18 to 40 years; no clinical or biochemical evidence of hypogonadism, thyroid dysfunction or hyperprolactinemia; no therapeutic or recreational drug use; no systemic disease co-morbidity. Additional inclusion criteria for women were as follows: regular menstrual cycles; no oral contraceptive pill therapy within the last year; no clinical or biochemical evidence of polycystic ovarian syndrome.

4.3.2 Kisspeptin-10 and -54 peptide synthesis

Human sequence kisspeptin-54 peptide was synthesised by Advanced Biotechnology Centre, Imperial College London. Human sequence kisspeptin-10 was synthesised by Bachem Holding AG (Bubendorf, Switzerland). Both peptides were purified by reverse-phase High Performance Liquid Chromatography (HPLC). Electrospray mass spectroscopy and amino acid analysis confirmed identity of the peptide. Toxicology testing in animals was conducted prior to administration to human volunteers. The Limulus amoebocyte lysate test (LAL) detected no endotoxin (Associates of Cape Cod, Liverpool, UK), and bacterial culture was sterile (Department of Microbiology, Hammersmith Hospital, London, UK), in samples of kisspeptin-10 and kisspeptin-54 peptide. Vials of freeze-dried kisspeptin-10 and kisspeptin-54 were stored at minus 20°C and reconstituted in 0.9% saline.
4.3.3 Study Days

Subjects were admitted to our clinical investigation unit and asked to lay supine for the duration of each study. Urine was tested to exclude pregnancy in women (Clearview Easy-HCG; Inverness Medical Innovations Inc. Waltham, MA). All blood samples were analysed for measurement of serum LH, FSH, oestradiol, testosterone, and plasma kisspeptin immunoreactivity (IR). Serum and plasma samples were stored at -20°C until analysis. Heart rate, blood pressure, and the presence of any symptoms were recorded at regular intervals (10-15 minutes). Women in the follicular phase of their menstrual cycle were studied between days 2-10 of their cycle. Women in the preovulatory phase were studied 15-16 days before the start of their next menstrual cycle.

4.3.4 Serum reproductive hormone measurement

Blood samples for serum LH, FSH and oestradiol analysis were collected in plain serum vacutainer tubes (Beckton Dickson, Franklin Lakes, NJ, USA). Samples were allowed to clot prior to centrifugation and separation of serum. Serum samples were stored at -20°C until analysis. LH, FSH, oestradiol and total testosterone were measured using automated chemiluminescent immunoassays (Abbott Laboratories, Abbott Park, IL). Reference ranges for males were as follows: LH, 4–14 IU/l; FSH, 1.5–8 IU/l; testosterone, 10–28 nmol/l. Reference ranges for females were as follows: LH 2–10 IU/L follicular, 20–60 IU/L midcycle, 4–14 IU/L luteal; FSH 10-50 IU/L, mid-cycle, 1.5–8 IU/L, follicular and luteal; oestradiol <300 pmol/L early follicular, 400-1500 pmol/L midcycle, 200-1000 pmol/L luteal. The respective intra- and inter-assay coefficients of variation for each assay were: LH 4.1% and 2.7%; FSH 4.1% and 3.0%; oestradiol 3.3% and 3.0%; total testosterone 4.2% and 2.8%. Analytical sensitivities were: LH 0.5 IU/l, FSH 0.05 IU/l; oestradiol 37 pmol/l; total testosterone 2 nmol/l.
4.3.5 **Kisspeptin measurement**

Blood samples for plasma kisspeptin analysis were collected in lithium heparin tubes (Beckton Dickson, Franklin Lakes, NJ, USA) containing 5000 kallikrein inhibitor units of aprotinin (0.2ml Trasylol; Bayer, Newbury, UK). Samples were immediately centrifuged at room temperature using a Hettich EBA 20 machine (Hettich International, Tuttingen, Germany) for 10 minutes at 3000rpm, and then separated. Plasma samples were stored at minus 20°C until analysis. Plasma kisspeptin immunoreactivity (IR) was measured using an in house radioimmunoassay. Antibody GQ2 was raised in sheep immunised with synthetic human kisspeptin-54 (Bachem UK Ltd.) conjugated to bovine serum albumin (BSA) by glutaraldehyde and used at a final dilution of 1:3,500,000. The antibody cross-reacted 100% with human kisspeptin-54, kisspeptin-14, and kisspeptin-10 and less than 0.01% with other related RF amide proteins, including prolactin-releasing peptide, RF amide-related peptide 1 (RFRP1, human and rat), RFRP2 (human), RFRP3 (human), QRFP43 (human), neuropeptide FF (human), and neuropeptide AF (human). The iodogen method was used to prepare the $^{125}\text{I}$-kisspeptin-54 label, which was subsequently purified by High Performance Liquid Chromatography (Salacinski et al. 1981). The assay was carried out in duplicate using dilutions of neat plasma in 0.7ml of 0.06M phosphate buffer (pH 7.2) containing 0.3% BSA. Incubation was for 3 days at 4°C. Subsequently free and antibody-bound label were separated by charcoal adsorption. The limit of detection was 2 pmol/l of plasma kisspeptin with 95% confidence interval, and the intra- and interassay coefficients of variation were 8.3% and 10.2%, respectively.
4.3.6 Study 1: The effects of intravenous bolus injection of saline or kisspeptin-10 in healthy male volunteers on plasma kisspeptin IR and reproductive hormones

Intravenous bolus injection of 0.9% saline or kisspeptin-10 (at doses 0.3, 1.0, 3.0, or 10 nmol/kg) was administered at time 0 min. For all studies the intravenous bolus was given via a cannulated antecubital vein over 10 seconds and subsequently flushed with 10ml of 0.9% saline. Blood samples were taken at -30, 0, 10, 20, 30, 40, 50, 60, 75, 90, 120, 150, and 180 minutes for measurement of serum reproductive hormones and plasma kisspeptin IR (n=4-5 per group).
4.3.7 Study 2: Effects of intravenous bolus injection of saline, kisspeptin-10 or kisspeptin-54 in healthy female volunteers

A: Follicular phase of the menstrual cycle

Women between day 2-10 of their menstrual cycle were administered an intravenous bolus injection of 0.9% saline, or kisspeptin-10 (at doses 1.0, 3.0, or 10 nmol/kg), or kisspeptin-54 (1.0 nmol/kg) at time 0 min, and blood samples were taken at -30, 0, 10, 20, 30, 40, 50, 60, 75, 90, 120, 150, and 180 min (n=4-5 per group).

B: Preovulatory phase of the menstrual cycle

Women 15-16 days before their next predicted period received intravenous bolus injection of 10 nmol/kg kisspeptin-10 as described for the follicular phase study above (n=5).

4.3.8 Study 3: Effects of subcutaneous bolus injection of saline or kisspeptin-10 on plasma kisspeptin-IR and serum reproductive hormones in healthy female volunteers in the follicular phase of menstrual cycle

Subjects in the follicular phase of menstrual cycle were admitted to our clinical investigation unit. Kisspeptin-10 (at doses of 2, 4, 8, 16, or 32 nmol/kg) or 0.9% saline were subcutaneously administered at time 0 minutes, and blood samples taken at -30, 0, 15, 30, 45, 60, 75, 90, 120, 150, 180, 210 and 240 minutes for measurement of serum LH, FSH oestradiol and plasma kisspeptin immunoreactivity (IR) (n=4-5 per group).

4.3.9 Study 4: Effects of intravenous infusion of saline or kisspeptin-10 on plasma kisspeptin-IR and serum reproductive hormones in healthy female volunteers

Subjects in the follicular phase of menstrual cycle were admitted to our Clinical Investigation Unit. Kisspeptin-10 was dissolved in saline containing gelofusine (5% vol/vol) (Braun Medical,
Sheffield, UK) to minimize peptide adsorption to the infusion system (Kraegen et al. 1975) and was infused over 90 minutes. During the first 30 minutes of infusion, the volunteers were infused with 20, 50, 90, 180, 360 or 720 pmol/kg/min. The infusion rate for each volunteer was then halved for the remaining 60 min of each infusion. This dosing regimen was designed to achieve a steady-state concentration of serum kisspeptin during the infusion period (Dhillo et al. 2005a; Edwards et al. 1999). Blood samples were taken at -30, 0, 15, 30, 45, 60, 75, 90, 120, 150, 180, 210 and 240 minutes for measurement of serum LH, FSH oestradiol and plasma kisspeptin IR.

4.3.9.1 Study 5: Pharmacokinetic profile of kisspeptin-IR during intravenous infusion of kisspeptin-10

To determine the plasma half-life of kisspeptin-10 in men, and in women during the follicular and preovulatory phases of the menstrual cycle, frequent blood sampling was performed during intravenous infusion of 360 pmol/kg/min of kisspeptin-10. Detailed blood sampling was performed at 1 minutely (from 91-100 min) and 2 minutely (from 102-120 min) intervals immediately after stopping the kisspeptin-10 infusion (at time 90 min). Blood samples were assayed for plasma kisspeptin IR. The linear regression line of natural log plasma kisspeptin IR was used to calculate the half-time of disappearance ($t_{1/2}$) for infused kisspeptin-10 in healthy males and females.

4.3.9.2 Data Analysis

Data are presented as mean ± SEM. Area under the curve (AUC) was calculated to provide a cumulative measure of kisspeptin/reproductive hormone change throughout the time course of the study. Time profiles of hormone levels were compared using two-way ANOVA with Bonferroni's multiple comparison test. Pairs of means were compared with unpaired $t$ tests, and multiple means of AUC reproductive hormone release were compared using one-
way ANOVA with Bonferonni’s multiple comparison test. Slopes of linear regression lines were compared using an $F$ test. Half-lives were calculated using natural log 2/gradient of linear regression line and compared using one-way ANOVA with Bonferonni’s multiple comparison test. For all statistical tests, $P < 0.05$ was considered statistically significant. All data of serum reproductive hormones during treatment are presented as increases in serum levels after injection when compared with pre-injection level.

### 4.4 Results

Baseline characteristics of the subjects recruited after medical screening are summarised in table 9 below. There was no significant difference in age or body mass index (BMI) between the male and female volunteers. In accordance with normal physiology circulating levels of LH, FSH, and oestradiol were significantly higher in the preovulatory phase of the menstrual cycle in females.
Table 9. Baseline characteristics of healthy male and female volunteers recruited to the study.

Data are shown as mean ± SEM.

<table>
<thead>
<tr>
<th>Baseline</th>
<th>Healthy male</th>
<th>Healthy female</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>28.8 ± 2.1</td>
<td>31.8 ± 1.4</td>
<td>0.23</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.5 ± 0.5</td>
<td>22.6 ± 0.8</td>
<td>0.09</td>
</tr>
<tr>
<td>Length of menstrual cycle (d)</td>
<td></td>
<td>28 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>LH (iU/l)</td>
<td>2.9 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicular</td>
<td>3.9 ± 0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preovulatory</td>
<td>28.0 ± 4.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FSH (iU/l)</td>
<td>2.6 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicular</td>
<td>3.8 ± 0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preovulatory</td>
<td>9.8 ± 0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Testosterone (nmol/l)</td>
<td>21.6 ± 1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oestradiol (pmol/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicular</td>
<td>228 ± 53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preovulatory</td>
<td>726 ± 71</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

P < 0.0001 vs. follicular phase of the menstrual cycle

P < 0.001 vs. follicular phase of the menstrual cycle
4.4.1 Study 1: Effects of IV bolus injection of saline or kisspeptin-10 in healthy male volunteers

Subjects reported no side effects following injection of kisspeptin-10 or saline. No significant changes in heart rate, or blood pressure were observed following kisspeptin-10 administration. Levels of plasma kisspeptin-IR were undetectable following saline injection. The plasma kisspeptin IR was elevated after intravenous bolus injection of kisspeptin-10 at all doses in healthy male volunteers (Figure 25 A, B). The peak occurred for all doses at 10 minutes after injection and was statistically significant (vs. saline) for 3 and 10 nmol/kg of kisspeptin-10 (Figure 25 A). Subsequently plasma kisspeptin IR returned to undetectable levels 50 minutes after injection. The highest plasma kisspeptin-IR was observed following intravenous bolus injection of 10 nmol/kg kisspeptin-10 (mean AUC kisspeptin-IR: 700 ± 160 h.pmol/l, P<0.001 vs. saline) (figure 25 B). At this dose, mean peak kisspeptin-IR (3350 ± 725 pmol/l) was observed 10 min post injection.
Figure 25. Plasma kisspeptin levels post intravenous bolus of saline or kisspeptin-10 in healthy men.

Plasma kisspeptin IR (A) after intravenous bolus injection of saline or 0.3, 1, 3, 10 nmol/kg kisspeptin-10. For 10 nmol/kg vs. saline: $P<0.001$. For 3 nmol/kg vs. saline: $P<0.001$. Mean kisspeptin IR AUC (B) post-intravenous injection of saline, 0.3, 1, 3, 10 nmol/kg kisspeptin-10 over the 180 min timecourse. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. 
Serum LH was significantly elevated after administration of each tested dose of intravenous bolus kisspeptin-10 compared with saline (Figure 26 A, B) from 20-120 minutes. Peak stimulation of serum LH was observed 30-50 min after injection which was 20-40 minutes after peak plasma kisspeptin IR. Thereafter serum LH levels returned to baseline 180 min after injection (Figure 26 A). Maximal stimulation of LH was observed after intravenous bolus of 10 nmol/kg kisspeptin-10 (mean AUC LH increase was 5.3 ± 1.2 h.IU/l, \( P<0.001 \) vs. saline), although there was significant increase for all doses (Figure 26 B).
Figure 26. Serum LH levels following intravenous bolus administration of saline or kisspeptin-10 in healthy men.

LH increase (A) after IVB injection of saline or 0.3, 1, 3, 10 nmol/kg kisspeptin-10. For 0.3 nmol/kg vs. saline: α, \( P<0.05 \); ααα, \( P<0.001 \). For 1 nmol/kg vs. saline: β, \( P<0.05 \); ββ, \( P<0.01 \); βββ, \( P<0.001 \). For 3 nmol/kg vs. saline: γγ, \( P<0.01 \); γγγ, \( P<0.001 \). For 10 nmol/kg vs. saline: δ, \( P<0.05 \); δδ, \( P<0.01 \), δδδ, \( P<0.001 \). Mean serum LH increase AUC (B) for saline, 0.3, 1, 3, 10 nmol/kg kisspeptin-10 post-intravenous bolus. *, \( P<0.05 \); **, \( P<0.01 \); ***, \( P<0.001 \).
Serum FSH was significantly increased compared with saline injection after intravenous bolus injection of 0.3 and 3.0 nmol/kg kisspeptin-10 (Figure 27 A, B) up to 90 minutes after injection. Peak FSH stimulation occurred slightly later than for LH, between 40-150 minutes after injection depending on dose (Figure 27 A). Maximal stimulation of FSH was observed after intravenous bolus of the smallest dose, 0.3 nmol/kg kisspeptin-10 (mean AUC FSH increase was 1.2 ± 0.5 h.iU/l, \( P<0.05 \) vs. saline), although significant stimulation was also seen at 3 nmol/kg (Figure 27 B).
Figure 27. Serum FSH levels following intravenous bolus (IVB) administration of saline or kisspeptin-10 in healthy men.

Serum FSH (A) after IVB of saline or 0.3, 1, 3, 10 nmol/kg kisspeptin-10. For 0.3 nmol/kg vs. saline: α, \( P<0.05 \); αα, \( P<0.01 \); ααα, \( P<0.001 \). For 3 nmol/kg vs. saline: γ, \( P<0.05 \); γγ, \( P<0.01 \); γγγ, \( P<0.001 \). Mean serum FSH increase AUC (B) for saline, 0.3, 1, 3, 10 nmol/kg kisspeptin-10 over the 180 min post-intravenous bolus. *, \( P<0.05 \); **, \( P<0.01 \); ***, \( P<0.001 \).
Serum testosterone AUC was significantly increased compared with saline injection after intravenous bolus injection of 1.0 nmol/kg kisspeptin-10 (Figures 28 B). Serum levels of testosterone at this dose steadily increased to peak levels 150-180 min after injection (Figure 28 A).
Figure 28 Serum testosterone levels following intravenous bolus (IVB) administration of saline or kisspeptin-10 in healthy men.

Serum Testosterone (A) after IVB of saline or 0.3, 1, 3, 10 nmol/kg kisspeptin-10. For 0.3 nmol/kg vs. saline: α, P<0.05. For 1 nmol/kg vs. saline: β, P <0.05; ββ, P<0.01. For 10 nmol/kg vs. saline: δ, P<0.05. Mean serum FSH increase AUC (B) for saline, 0.3, 1, 3, 10 nmol/kg kisspeptin-10 over the 180 min post-intravenous bolus. *, P<0.05; **, P<0.01; ***, P<0.001.
4.4.2 Study 2: Effects of intravenous bolus injection of saline, kisspeptin-10 or kisspeptin-54 in healthy female volunteers

A: Follicular phase of the menstrual cycle

The plasma kisspeptin IR was elevated after intravenous bolus injection of kisspeptin-10 at all doses in healthy female volunteers during the follicular phase of the menstrual cycle (Figure 29 A, B). The highest plasma kisspeptin IR was observed after intravenous bolus injection of 10 nmol/kg kisspeptin-10 (mean AUC kisspeptin in healthy females during the follicular phase was 527 ± 108 h.pmol/l, \( P < 0.001 \) vs. saline). Although this was lower when compared with kisspeptin IR after the same dose of kisspeptin-10 to men (700 ± 160 h.pmol/l for men), this difference was not statistically significant (\( P = 0.42 \) vs. men). At the dose of 10 nmol/kg kisspeptin-10, mean peak kisspeptin IR (2638 ± 302 pmol/l) was observed 10 min after injection. Plasma kisspeptin IR subsequently returned to undetectable levels 50 min after injection (Fig 29 A).

Kisspeptin-54 was also administered as an IV bolus in this study as a positive control as previous work has demonstrated that it stimulates reproductive hormones in females. Peak kisspeptin IR occurred 30-40 minutes after injection of kisspeptin-54 in contrast to kisspeptin-10 which peaked at 10 minutes after injection (Figure 29 A).
Figure 29 Plasma kisspeptin levels following intravenous bolus (IVB) administration of saline or kisspeptin-10 in healthy women.

Plasma kisspeptin IR (A) after IVB of saline, 1, 3, 10 nmol/kg kisspeptin-10 and 1 nmol/kg kisspeptin-54 in follicular or preovulatory phase. For 3 nmol/kg vs. saline: φφφ, \( P < 0.001 \). For 10 nmol/kg follicular vs. saline: λλλ, \( P < 0.001 \). For 10 nmol/kg preovulatory vs. saline: µµµ, \( P < 0.001 \). For kisspeptin-54 (1nmol/kg) vs. saline: ϑ, \( P < 0.05 \); ϑϑϑ, \( P < 0.001 \). Mean kisspeptin IR AUC (B) for saline, 1, 3, 10 nmol/kg kisspeptin-10 and 1 nmol/kg kisspeptin-54 post-intravenous bolus in follicular and preovulatory phase. *, \( P < 0.05 \); **, \( P < 0.01 \); ***, \( P < 0.001 \).
Unlike in the male study, no significant changes in serum LH, FSH or oestradiol were observed after intravenous bolus injection of kisspeptin-10 at all doses tested in the follicular phase (Figures 30, 31, 32). However mean LH and FSH AUC were significantly elevated after intravenous bolus injection of 1 nmol/kg of kisspeptin-54 (Figures 30, 31).

**B: Preovulatory phase of the menstrual cycle**

Kisspeptin IR was significantly elevated in women during the preovulatory phase after kisspeptin-10 injection compared with saline. This elevation was not significantly different when compared with kisspeptin IR after the same dose of kisspeptin-10 in follicular phase women or men (mean AUC kisspeptin IR in preovulatory phase was 320 ± 56 h.pmol/l, \( P = 0.13 \) vs. follicular phase and \( P = 0.06 \) vs. men) (Figure 29).

Serum LH and FSH were both significantly elevated after intravenous bolus injection of 10 nmol/kg kisspeptin-10 in female volunteers during the preovulatory phase of the menstrual cycle (mean AUC increase was 30.4 ± 11.1 h.iU/l (LH), \( P < 0.05 \) vs. saline, and 6.9 ± 0.9 h.iU/l (FSH), \( P < 0.01 \) vs. saline) (Figures 30, 31). This was in contrast to the lack of response seen in the follicular phase. Peak LH and FSH stimulation occurred 40 minutes after injection of kisspeptin-10 with significant elevation maintained until 75 minutes (Figures 30, 31).

Serum oestradiol, however, was not altered significantly by kisspeptin-10 in the preovulatory phase (mean AUC oestradiol increase was 111 ± 96 h/pmol/l, \( P = 0.13 \) vs. saline) (Figure 32).
Figure 30. Serum LH levels following intravenous bolus (IVB) administration of saline or kisspeptin-10 in healthy women.

Serum LH (A) after intravenous bolus injection of saline, 1, 3, 10 nmol/kg kisspeptin-10 and 1 nmol/kg kisspeptin-54 in follicular or preovulatory phase. For 10 nmol/kg preovulatory vs. saline: µµ, \( P<0.01 \); µµµ, \( P<0.001 \). Mean serum LH AUC (B) for saline, 1, 3, 10 nmol/kg kisspeptin-10 and 1 nmol/kg kisspeptin-54 over the 180 min timecourse post-intravenous bolus in follicular and preovulatory phase. *, \( P<0.05 \); **, \( P<0.01 \); ***, \( P<0.001 \).
Figure 31. Serum FSH levels following intravenous bolus (IVB) administration of saline or kisspeptin-10 in healthy women.

Serum FSH increase (A) after IVB of saline, 1, 3, 10 nmol/kg kisspeptin-10 and 1 nmol/kg kisspeptin-54 in follicular or preovulatory phase. For 10 nmol/kg preovulatory vs. saline: µ, \( P<0.05 \); µµ, \( P<0.01 \); µµµ, \( P<0.001 \). Mean serum FSH AUC (B) for saline, 1, 3, 10 nmol/kg kisspeptin-10 and 1 nmol/kg kisspeptin-54 post-intravenous bolus in follicular and preovulatory phase. *, \( P<0.05 \); **, \( P<0.01 \); ***, \( P<0.001 \).
Figure 32. Serum oestradiol levels following intravenous bolus (IVB) administration of saline or kisspeptin-10 in healthy women.

Serum oestradiol increase (A) after IVB injection of saline, 1, 3, 10 nmol/kg kisspeptin-10 and 1 nmol/kg kisspeptin-54 in follicular or preovulatory phase. Mean serum oestradiol AUC (B) for saline, 1, 3, 10 nmol/kg kisspeptin-10 and 1 nmol/kg kisspeptin-54 post-intravenous bolus in follicular and preovulatory phase.
4.4.3 Study 3: Effects of subcutaneous bolus injection of saline or kisspeptin-10 on plasma kisspeptin-IR and serum reproductive hormones in healthy female volunteers

Subjects reported no side effects following subcutaneous injection of kisspeptin-10 or saline. No significant changes in heart rate, systolic or diastolic blood pressure were observed following kisspeptin-10 administration. Plasma kisspeptin-IR in healthy female volunteers was significantly raised during the 4 hours following subcutaneous (sc) injection of kisspeptin-10 at doses of 4 nmol/kg and higher, when compared with saline (Figure 33 A, B). Progressively higher plasma kisspeptin-IR was observed with each increment in sc dose of kisspeptin-10 injection. The highest plasma kisspeptin-IR following sc injection was observed following injection of 32 nmol/kg kisspeptin-10 (mean AUC kisspeptin-IR, 201 ± 16h.pmol/l, P<0.001 vs. saline). No significant changes in serum reproductive hormone levels were observed following sc bolus injection of kisspeptin-10 at any dose (Figure 34 A-D).
Figure 33. Plasma kisspeptin immunoreactivity (IR) following subcutaneous bolus (sc) injection of kisspeptin-10 to healthy women.

Time profiles (A) and area under curve AUC for plasma kisspeptin IR (B) during 4 hours after sc bolus injection of saline or kisspeptin-10 (n=4-5 per group). Data is shown as mean +/- SEM. *P<0.05; **P<0.01; ***P < 0.001
Figure 34. Serum reproductive hormone levels following subcutaneous bolus injection of kisspeptin-10 to healthy women.

Time profiles and area under curve (AUC) changes in serum LH (A, B) and FSH (C, D) during 4 hours after sc bolus injection of saline or kisspeptin-10 (n=4-5 per group). Data is shown as mean +/- SEM. *P<0.05; **P<0.01; ***P < 0.001

![Graph A: LH increase vs time](image)

![Graph B: AUC LH increase vs dose](image)
C

Injection Time (minutes)
FSH increase (IU/L)

D

Dose of kisspeptin-10 (nmol/kg)
AUC FSH increase (h.IU/L)
4.4.4 Study 4: Effects of intravenous infusion of kisspeptin-10 on plasma kisspeptin-IR and serum reproductive hormones in healthy female volunteers

Ninety minute intravenous infusions of kisspeptin-10 were administered to healthy female volunteers in the follicular phase of menstrual cycle at doses 20, 50, 90, 180, 360 or 720 pmol/kg/min. Subjects reported no side effects during infusion of kisspeptin-10. No significant changes in heart rate, systolic or diastolic blood pressure were observed during kisspeptin-10 infusion. Plasma kisspeptin-IR increased during intravenous infusion of kisspeptin-10 at all doses (Figure 35 A). The highest plasma kisspeptin-IR was observed during intravenous infusion of 720 pmol/kg/min kisspeptin-10 (mean AUC kisspeptin-IR, 2518 ± 100 h.pmol/l). All intravenous infusion doses of kisspeptin-10 were associated with a higher mean plasma kisspeptin-IR than the highest studied sc dose of kisspeptin-10 (32 nmol/kg, mean AUC kisspeptin-IR 201 ± 16 h.pmol/l) (figure 35 B). No significant changes in serum reproductive hormone levels were observed for any dose of kisspeptin-10 infusion, in healthy female volunteers in the follicular phase of menstrual cycle (Figure 36 A-C).
Figure 35. Plasma kisspeptin immunoreactivity (IR) during a 90 minute intravenous infusion of kisspeptin-10 to healthy women.

Time profiles (A) and area under curve AUC (B) for plasma kisspeptin IR. Data is shown as mean +/- SEM *P<0.05; **P<0.01; ***P < 0.001
Figure 36. Serum reproductive hormone levels during intravenous infusion of kisspeptin-10 to healthy women.

Area under the curve AUC changes in serum LH (A), FSH (B) and oestradiol E2 (C) during 4 hours after commencement of a 90 minute intravenous infusion of kisspeptin-10 in the follicular phase of menstrual cycle (n=4-5 per group). Data is shown as mean +/- SEM. **P < 0.01; ***P < 0.001.
4.4.5 Study 5: Pharmacokinetic profile of kisspeptin-IR during intravenous infusion of kisspeptin-10

To determine the plasma half-life of kisspeptin-10, detailed blood sampling was performed in men and women during the follicular and preovulatory phases of the menstrual cycle, during the 4 hours after commencing an intravenous infusion of 360 pmol/kg/min of kisspeptin-10. Peak mean kisspeptin-IR was observed 30 minutes after commencing kisspeptin-10 infusion (1088 ± 243 pmol/l). Plasma kisspeptin-IR fell rapidly nearly ten-fold within 30 minutes of cessation of the kisspeptin-10 infusion (Figures 37, 38). The plasma half-life of kisspeptin-10 was calculated to be 3.3 minutes. During the infusion males had higher plasma kisspeptin IR than females in both the follicular and preovulatory phases.
Figure 37. Plasma kisspeptin IR before, during and after a 90 minute infusion of 360 pmol/kg/min kisspeptin-10.

Blood was sampled every 1 min between 90-100 min and then every 2 min between 100-120 min. For men vs. women during the follicular phase: ψ, P<0.05; ψψ, P<0.01; ψψψ, P<0.001. For men vs. women during the preovulatory phase: ω, P<0.05; ωωω, P<0.001.
When plotted on a natural log scale, linear regression slopes were not significantly different among the three groups ($F = 0.013$; Degrees of Freedom $= 2$; Degrees of Freedom denominator $= 137$; $P = 0.88$). Plasma half-lives of kisspeptin-10 were calculated using these regression slopes and were also statistically similar between all three groups: male, $4.62 \pm 0.34$ min; female follicular phase $4.72 \pm 0.43$ min; female preovulatory phase $4.40 \pm 0.34$ min (Fig 38).
Figure 38. Natural Logarithm kisspeptin IR against time immediately after cessation of a 90 minute infusion of 360 pmol/kg/min kisspeptin-10.

Vertical detached lines indicate calculated half-lives for females in the follicular and preovulatory phase and males.
5. Discussion

This study reveals a previously unknown sexual dimorphism in responsiveness to kisspeptin-10, by intravenous bolus injection in healthy men and women. Numerous studies in various male and female animal species have demonstrated that kisspeptin-10 robustly stimulates gonadotrophin release. Thus kisspeptin signalling is a potential therapeutic target for treating reproductive disorders (Arreguin-Arevalo et al. 2007; Caraty et al. 2007; Irwig et al. 2004; Kadokawa et al. 2008; Lents et al. 2008; Navarro et al. 2004; Plant et al. 2006; Ramaswamy et al. 2007; Shahab et al. 2005). Studies on the effects of kisspeptin 10 on male primate models have demonstrated gonadotrophin stimulation (Plant et al. 2006; Ramaesh et al. 2010; Shahab et al. 2005). Central administration of kisspeptin10 into the stalk median eminence/medial basal hypothalamus stimulates release of GnRH (Keen et al. 2008; Roseweir et al. 2009). Two studies on the effects of kisspeptin-10 on healthy men have also demonstrated that kisspeptin-10 stimulates gonadotrophin release (Chan et al. 2011; George et al. 2011). A recent study in pubertal and prepubertal female monkeys has demonstrated that central administration of kisspeptin-10 stimulated GnRH release in both groups in a dose dependent fashion (Guerriero et al. 2012).

I observed that an intravenous bolus injection of kisspeptin-10 significantly stimulated LH release in healthy men at all doses tested. These results are consistent with previous reports on the effects of the administration of kisspeptin-54 (Dhillon et al. 2005) and kisspeptin-10 (Chan et al. 2011; George et al. 2011) on the HPG axis in healthy men.

I noted that intravenous bolus doses of kisspeptin-10 ranging from 0.3 – 10 nmol/kg were associated with similar degrees of gonadotrophin secretion in healthy male subjects. George et al. report that peripheral intravenous administration of kisspeptin-10, stimulates serum LH secretion at doses as low as 0.01 μg/kg with a maximal response seen at 1 μg/kg (George et al. 2011). Therefore, the doses of kisspeptin-10 selected during my study may have
stimulated near-maximal levels of gonadotrophin secretion in healthy men. Tachyphylaxis was noted in the George et al study; with increases in serum LH at higher kisspeptin doses of 3 μg/kg being lower than those stimulated by 1 μg/kg of kisspeptin-10 (George et al. 2011). This is consistent with previous reports of tachyphylaxis occurring in women after chronic kisspeptin-54 injections (Jayasena et al. 2009). Thus, the similarity between LH responses at all doses between 0.3 and 10 nmol/kg kisspeptin-10 in men might be explained in part by tachyphylaxis to kisspeptin-10 at the higher tested doses. Additional studies are required to investigate these observations.

Surprisingly, intravenous bolus injection of kisspeptin-10 failed to stimulate reproductive hormone release in healthy female volunteers during the follicular phase of menstrual cycle. Plasma kisspeptin IR was raised for only 40 min following intravenous bolus injection of kisspeptin-10 to male and female volunteers. Our previous clinical studies suggest that sc bolus injection of kisspeptin-54 raises kisspeptin IR for a longer period of time when compared with intravenous administration of kisspeptin-54. I therefore studied the effects of sc bolus injection of kisspeptin-10 in order to determine if a more sustained exposure to exogenous kisspeptin-10 would stimulate reproductive hormone release in healthy women. Despite elevations in plasma kisspeptin IR for up to 90 min post-injection, serum gonadotrophin levels were not elevated following sc bolus injection of kisspeptin-10 in healthy women in the follicular phase of menstrual cycle.

In order to study the effects of sustained, high dose kisspeptin-10 administration on reproductive hormone release, I then administered intravenous infusions of kisspeptin-10 to healthy women. Despite markedly raised plasma levels of kisspeptin IR up to 2000pmol/l, serum gonadotrophin levels were not elevated during intravenous infusion of kisspeptin-10 to healthy female volunteers in the follicular phase of menstrual cycle. Our data therefore suggest that intravenous bolus injection, subcutaneous bolus injection, or intravenous infusion of kisspeptin-10 fails to stimulate gonadotrophin release in healthy women during the follicular phase of menstrual cycle, at the doses tested in this study. This is the first study
to investigate the effects of kisspeptin-10 in women. This study demonstrates that women in the follicular phase of menstrual cycle are less responsive to kisspeptin-10 administration than men.

Only a marginal elevation of plasma kisspeptin IR (approximately 10 h.pmol/l) was necessary to stimulate significant LH secretion in men after injection of kisspeptin-10 (0.3 nmol/kg iv bolus); by contrast, a 50-fold greater elevation in plasma kisspeptin IR failed to stimulate LH release in women given the high dose 10 nmol/kg kisspeptin-10 during the follicular phase. Furthermore the doses used in the study produced similar cumulative plasma kisspeptin-IR in both males and females ($P = 0.42$ for 10 nmol/kg kisspeptin-10 in men vs. women). It may be expected, therefore that levels of kisspeptin-10 may have been modified by factors known to differ between the sexes, such as body fat content or clearance of the peptide from the plasma (Soldin, Chung, & Mattison 2011). However this study also demonstrated no differences in plasma half-life and linear regression gradient for kisspeptin-10 in males and females.

Consistent with our previous studies, (Dhillon 2007, Jayasena et al. 2009, Jayasena 2010) kisspeptin-54 was able to significantly stimulate LH and FSH increases in women. Previous studies have suggested that kisspeptin 54 has a higher in vivo potency for stimulating the kisspeptin receptor. Jayasena et al. reported an increased potency of kisspeptin-54 over kisspeptin-10 (Jayasena et al. 2011) for stimulating LH in female adult rats. It has also been previously described that kisspeptin-52 (Irwig et al. 2004) and kisspeptin-54 (Thompson et al. 2004) stimulate LH more potently when compared with kisspeptin10 in male rats. Therefore our findings may be explained by an increased potency of kisspeptin 54 compared to kisspeptin 10. However, a ten-fold increase in molar dose for kisspeptin-10 compared to kisspeptin-54 still failed to stimulate reproductive hormone release in women in the follicular phase on the menstrual cycle in this study.
This difference between kisspeptin-10 and kisspeptin-54 on gonadotrophin release may be a consequence of rapid breakdown of kisspeptin-10 in the circulation. Indeed the in vivo plasma half-life of iv kisspeptin-10 in this study was calculated as approximately 4 minutes, which is 7-fold shorter than the calculated in vivo plasma half-life of kisspeptin-54; which is 28 minutes (Dhill et al. 2005). It is possible that the kisspeptin receptor requires a prolonged exposure to raised plasma kisspeptin levels for activation. However, the administration of a subcutaneous dose of kisspeptin 10 or a 90 minute infusion of kisspeptin-10; which resulted in a similar time-profile of plasma kisspeptin IR as that seen with kisspeptin-54, also failed to cause reproductive hormone stimulation.

Women in the preovulatory phase of the menstrual cycle are known to be significantly more sensitive to the effects of kisspeptin-54 on gonadotrophin release when compared with women in the follicular phase of the menstrual cycle (Dhill et al. 2007). In keeping with this observation, an intravenous bolus injection of kisspeptin-10 significantly stimulated LH and FSH release in women during the preovulatory phase of the menstrual cycle in this study. As the pharmacokinetic profiles of plasma kisspeptin IR in both the follicular and preovulatory phases were similar, these results suggest that as with kisspeptin-54, women have heightened sensitivity to kisspeptin-10 during the preovulatory phase of the menstrual cycle. This suggests that the different background hormonal milieu with higher oestradiol levels in the pre-ovulatory phase may influence the responsiveness to kisspeptin-10.

A recent study, published after the completion of my work, examined the effects of kisspeptin-10 in women at different stages of the menstrual cycle and supports my findings. In this study the authors also demonstrate a difference in response to an intravenous bolus of kisspeptin-10 across the menstrual cycle, with stimulation in gonadotrophin release in the luteal and preovulatory phase and a minimal response seen in the follicular phase (Chan et al. 2012). In this study the authors report that an intravenous bolus dose of 0.24 nmol/kg of kisspeptin-10 consistently stimulated a significant LH pulse in the preovulatory and luteal phase, with the response in the preovulatory phase being significantly higher. However,
despite increasing the administered dose of kisspeptin-10 to 0.72 nmol/kg no consistent response in LH stimulation was noted in the follicular phase (Chan et al. 2012). The authors suggest that GnRH neurones may have varied responsiveness to exogenous kisspeptin stimulation across the menstrual cycle. They postulate a theory that GnRH neurones are maximally stimulated by endogenous kisspeptin in the follicular phase and thus the additional ability to respond to exogenous kisspeptin is limited. Further studies are required to investigate this hypothesis.

Studies in rodents have identified two distinct kisspeptin neuronal populations that have different responses to oestradiol. In female rodents, c-fos expression within kisspeptin neurones and levels of KISS1 expression are increased within the AVPV nucleus of the hypothalamus immediately preceding ovulation as a result of positive feedback on this nucleus by oestradiol (Smith et al. 2006). This contrasts against the negative feedback by oestradiol on a different distinct population of kisspeptin neurones in the ARC as evidenced by reduced levels of ARC KISS1r expression in rat hypothalamic fragments at proestrus when compared with diestrus (Roa et al. 2006). This suggests a complex interplay between AVPV and ARC neurones with GnRH neurones in rodents.

*KISS1* expressing neurones have only been identified in humans in the infundibular nucleus and to a lesser extent the medial preoptic area (Rometo et al. 2007). These neurones express the oestrogen receptor α (ERα). GnRH neurones express ERβ, but this is not involved in feedback mechanisms and hence it is thought that gonadal steroid feedback on the hypothalamus in the reproductive axis is via the kisspeptin neurones. In the low oestrogen state of menopause there is significant increase in *KISS1* expression in the infundibular nucleus together with hypertrophy and increased numbers of these *KISS1*-expressing neurones when compared to pre-menopausal specimens (Rance, 2009). Hence this suggests that higher oestrogen states may downregulate *KISS1* expression in humans. However, to explain the preovulatory surge of gonadotrophins there is likely to be a currently unidentified subpopulation of kisspeptin neurones in women that are stimulated by increased
oestrogen levels. To explain the results of the current study it is possible that there is a complex interaction dependent on background oestradiol levels between these kisspeptin neurones, exogenous kisspeptin-10 and the kisspeptin receptor of the GnRH neurones.

Guerriero et al. report that in female rhesus monkeys low doses of kisspeptin 10 (10nM) stimulated larger GnRH responses in pubertal monkeys compared to prepubertal monkeys (Guerriero, Keen, & Terasawa 2012). The authors note that circulating oestradiol levels are higher in pubertal monkeys and that ovariectomy in these animals, which reduces oestradiol levels, eliminated the GnRH response to low and high doses of kisspeptin-10. Thus the difference in response to kisspeptin appears to be related to oestradiol levels. However, the replacement of oestradiol in pubertal monkeys who had undergone ovariectomy resulted in only a partial restoration of the GnRH response to kisspeptin-10. The authors postulate whether this may be due to absence of other ovarian hormones, or failure in adequate amount and length of oestradiol replacement or a change in properties of the oestrogen receptor post ovariectomy (Guerriero et al. 2012).

In this study I noted that in both men and preovulatory women, kisspeptin-10 stimulated LH secretion more potently than FSH. This is consistent with previous studies of the effects of kisspeptin-10 and kisspeptin-54 in healthy men and women (Chan et al. 2011; Dhillo et al. 2005; Dhillo et al. 2007; George et al. 2011). In addition, in men the peak FSH increase occurred slightly later than the peak LH increase following iv bolus of kisspeptin-10 (40-150 min for FSH vs. 30-50 min for LH). This difference has previously been observed for both males and females (Dhillo et al. 2005; Dhillo et al. 2007). This study therefore suggests that both kisspeptin-10 and kisspeptin-54 stimulate LH secretion more potently and more rapidly than FSH. Administration of GnRH demonstrates a similar pattern of differing LH/FSH potency and rapidity of effect. Combining this with the additional similarity that LH and FSH responses to GnRH are also heightened in the pre-ovulatory phase, provides further evidence to suggest that GnRH is the intermediary messenger for kisspeptin to stimulate the anterior pituitary production of LH and FSH.
I did not observe any consistent stimulation of testosterone secretion after iv bolus kisspeptin-10 injection in healthy men when compared with the robust increases in serum LH and FSH observed at all tested doses. Significant increases in serum testosterone were observed only at 1.0 nmol/kg iv bolus kisspeptin-10, but these rises were marginal (no more than 10 h.nmol/l above baseline). Similarly, iv bolus injection of kisspeptin-10 stimulated gonadotropin release in women during the preovulatory phase of the menstrual cycle but did not increase serum oestradiol during 3 h after injection. Previous data suggest that at least 4 hours are required for serum levels of sex steroids to peak after a sc bolus injection of kisspeptin-54 (Dhillo et al. 2005; Dhillo et al. 2007; Jayasena et al. 2009). A longer period of blood sampling after injection may have revealed more pronounced alterations in sex steroid secretion in subjects after injection of kisspeptin-10. Alternatively it is possible that an iv bolus injection of kisspeptin-10 and kisspeptin-54 have a duration of action inadequate to stimulate significant gonadal sex steroid release.

In summary, this is the first clinical study to compare the effects of kisspeptin-10 administration on reproductive hormone release in healthy men and women. Kisspeptin-10 robustly stimulates gonadotrophin release in men and women in the preovulatory phase but fails to stimulate gonadotrophin release in the follicular phase. This sexual dimorphism as well as the different responses between the follicular and preovulatory phase have important clinical implications for the potential therapeutic use of kisspeptin-10 or kisspeptin-54 to treat disorders of reproduction and suggest that kisspeptin-54 may offer a more potent alternative.
Chapter 5

General Discussion
Since 2003 and the discovery of the role of kisspeptin in hypothalamo-pituitary-gonadal function our understanding of reproductive physiology has been revolutionised. Inactivating mutations of KISS1R in humans and rodents results in hypogonadotrophic hypogonadism and pubertal failure (de Roux et al. 2003; Seminara et al. 2003). Activating mutations of the KISS1R result in the clinical phenotype of central precocious puberty (Teles et al. 2008).

The novel finding that administration of kisspeptin to healthy men and women stimulates gonadotrophin hormone release (Dhillo et al. 2005; Dhillo et al. 2007), has revealed the potential of kisspeptin as a therapeutic agent for those patients with disorders of reproduction. In this thesis I report the first clinical studies examining the acute effects of administration of kisspeptin-54 on the reproductive axis in women with infertility due to hypothalamic amenorrhoea; as well as the effects of chronic administration of kisspeptin-54. I also report on the original discovery of sexual dimorphism in gonadotrophin response to exogenous administration of kisspeptin-10.

Hypothalamic amenorrhoea is a common cause of infertility, accounting for 30% of cases of amenorrhoea (Reindollar 1986). Hypothalamic amenorrhoea results from a failure of pulsatile GnRH secretion from the hypothalamus and may be triggered by energy deficits as well as psychological stress (Berga et al. 1989). In this thesis I report that exogenous administration of kisspeptin-54 results in an acute stimulation of serum levels of LH and FSH in women with hypothalamic amenorrhoea. Women with hypothalamic amenorrhoea appeared to be more sensitive to the effects of kisspeptin on gonadotrophin stimulation, with a 4 fold greater response, compared to previously published data on kisspeptin administration to healthy women. Castellano et al. reported that negative energy balance due to short term fasting in rats results in a decrease in hypothalamic expression of Kiss-1 mRNA and an increased hypothalamic expression of kisspeptin receptor GPR54 mRNA (Castellano et al. 2005). In vitro and rodent in vivo experiments have demonstrated an increased sensitivity to the effects of kisspeptin-10 administration on GnRH secretion and gonadotrophin stimulation in fasted animals (Castellano et al. 2005). Our finding of increased
sensitivity to the effects of kisspeptin on the HPG axis, in women with hypothalamic amenorrhoea, is consistent with these animal model data.

Although acute administration of kisspeptin has been shown to stimulate gonadotrophin release in healthy men and women (Dhillo et al. 2005; Dhillo et al. 2007), there have been no previous studies conducted on the effects of long-term chronic administration of kisspeptin in humans. I report that twice daily injections of kisspeptin-54 6.4 nmol/kg to women with hypothalamic amenorrhoea for two weeks results in tachyphylaxis. The reproductive hormone response to kisspeptin was much lower following the last kisspeptin injection compared to the first kisspeptin injection. In order to investigate whether the desensitisation to the effects of kisspeptin was due to desensitisation at the level of the hypothalamus on GnRH secretion or whether there was a decrease in sensitivity of the pituitary gonadotrophs to GnRH; I conducted a second experiment in which I investigated the effects of exogenous GnRH administration on gonadotrophin release following chronic kisspeptin administration. Despite losing sensitivity to the effects of kisspeptin, women with hypothalamic amenorrhoea treated with twice daily injections of kisspeptin-54, remained responsive to the gonadotrophin stimulation with GnRH. Thus, tachyphylaxis to the effects of kisspeptin administration appears to be upstream of the GnRH receptor probably at the level of hypothalamic KISS1R rather than at the pituitary gonadotrophs.

Continuous administration of kisspeptin has been shown to result in desensitisation of the effects of kisspeptin on gonadotrophin stimulation in monkeys and rodents (Seminara et al. 2006; Thompson et al. 2006; Roa et al. 2008). Delayed desensitisation of the KISS1R due to an Arg386Pro mutation, results in the clinical phenotype of precocious puberty (Teles et al. 2008). In vitro studies measuring total inositol phosphate production as a marker of KISS1R signalling, have demonstrated that the KISS1R is desensitised in a time dependent manner (Bianco et al. 2011). A proportion of internalised KISS1R is recycled and the remainder degraded by proteasomes, which results in long term desensitisation of the KISS1R (Bianco et al. 2011). The Arg386Pro mutation associated with precocious puberty results in reduced
degradation of the KISS1R resulting in an increased responsiveness to kisspeptin (Bianco et al. 2011).

Although I did not administer kisspeptin-54 as a continuous infusion, the twice daily injections did lead to raised plasma kisspeptin IR for 6 hours post injection resulting in prolonged exposure to elevated levels of kisspeptin. Therefore a different study protocol of kisspeptin-54 administration i.e. a lower dose or reduced frequency of injections may reduce the desensitisation seen in stimulation of gonadotrophin release. My subsequent studies went on to investigate the time course of the desensitisation of gonadotrophin response and the effects of different dosing regimens of kisspeptin to women with hypothalamic amenorrhoea. I found that tachyphylaxis of LH response to twice-daily sc kisspeptin-54 6.4 nmol/kg occurred gradually over the 14 day injection period, but FSH responsiveness reduced much more rapidly.

The studies comparing different dosing regimens of kisspeptin-54 showed that once- and twice-daily administration of kisspeptin-54 led to significant desensitisation of gonadotrophin responses in women with HA. The tachyphylaxis in response to chronic administration of kisspeptin-54 may be utilised for its clinical effects on down regulating the HPG axis. It has been suggested that chronic kisspeptin administration may be a useful a therapy for sex hormone responsive cancers (Seminara et al. 2006). In addition, pharmacological blockade of kisspeptin signalling may suppress LH pulsatility rather than basal LH levels (Roseweir et al. 2009), posing the potential development of a contraceptive therapy.

My studies demonstrated that twice-weekly administration of 6.4 nmol/kg kisspeptin-54 was only associated with partial desensitisation of gonadotrophin responses during a 2 week pilot study. I therefore conducted a randomised placebo controlled double blind study of twice weekly kisspeptin-54 6.4 nmol/kg administration to women with hypothalamic amenorrhoea. The findings from this study showed that over the study period of eight weeks participants remained responsive to kisspeptin with a partially sustained gonadotrophin response. There
were no adverse effects noted. Although there was stimulation of gonadotrophin release, on review of pelvic ultrasounds there was no significant changes in follicle growth in those women who received kisspeptin-54. This failure in stimulating follicle growth may be secondary to a reduced FSH response to kisspeptin compared to LH. Previous studies in healthy men and women have demonstrated that kisspeptin-54 administration does stimulate LH and FSH, but that the FSH reponse is lower (Dhillo et al. 2005; Dhillo et al. 2007). In addition my results demonstrate that the FSH responses to kisspeptin-54 desensitised more rapidly following twice-daily kisspeptin-54 injection than LH responses. Thus, FSH stimulation on the gonads may not have been high enough in amplitude or pulsatility to stimulate follicle growth.

Kisspeptin-10 is a shorter peptide produced by protelytic processing of a 145-amino acid precursor protein encoded by the KISS1 gene (Kotani et al. 2001). Previous studies have demonstrated that central or peripheral administration of kisspeptin-10 to a number of mammalian species stimulates gonadotrophin release (Irwig et al. 2004; Navarro et al. 2004; Gottsch et al 2004; Messager et al. 2005; Caraty et al. 2007; Plant et al. 2006; Ramaswamy et al. 2007; Shahab et al. 2005; Grieves et al. 2007; Lents et al. 2008; Kadokawa et al. 2008). The stimulatory effects of kisspeptin-10 on gonadotrophin release are inhibited by the central administration of a GnRH antagonist. Compared to kisspeptin-54, kisspeptin-10 has a shorter half-life and faster onset of action after intravenous administration in rodents (Mikkelsen et al. 2008;Mikkelsen et al. 2009). As kisspeptin-10 is simpler and thus cheaper to manufacture, future kisspeptin-based reproductive therapies may be based upon kisspeptin-10 rather than kisspeptin-54. Human male studies have demonstrated that an intravenous bolus injection of kisspeptin-10 potently stimulates LH secretion while a continuous infusion of kisspeptin-10 also increases testosterone, LH pulse frequency and amplitude (George et al. 2011, Chan et al. 2011). I carried out the first study comparing the effects of kisspeptin-10 administration in women versus men.
I report a previously unknown sexual dimorphism in responsiveness to kisspeptin-10 in healthy men and women. I observed that an intravenous bolus injection of kisspeptin-10 significantly stimulated LH release in healthy men. These results are consistent with previous reports on the effects of the administration of kisspeptin-54 (Dhillo et al. 2005) and kisspeptin-10 (Chan et al. 2011; George et al. 2011) on the HPG axis in healthy men. However, an intravenous bolus injection of kisspeptin-10 failed to stimulate reproductive hormone release in healthy female volunteers during the follicular phase of menstrual cycle.

In order to assess whether a more sustained exposure to exogenous kisspeptin-10 would stimulate reproductive hormone release in healthy women I studied the effects of subcutaneous bolus administration of kisspeptin-10 in addition to an intravenous bolus administration. Despite elevations in plasma kisspeptin IR for up to 90 min post-injection, serum gonadotrophin levels were not elevated by sc bolus injection of kisspeptin-10 in healthy women in the follicular phase of menstrual cycle.

I then went on to investigate the effects of administering intravenous infusion of kisspeptin-10 to healthy women to study the effects of sustained, high dose kisspeptin-10 administration on reproductive hormone release. Despite markedly raised plasma levels of kisspeptin IR up to 2000pmol/l, serum gonadotrophin levels were not elevated during intravenous infusion of kisspeptin-10 to healthy female volunteers in the follicular phase of menstrual cycle.

My data therefore demonstrates that intravenous bolus injection, subcutaneous bolus injection, or intravenous infusion of kisspeptin-10 fails to stimulate gonadotrophin release in healthy women during the follicular phase of menstrual cycle. This study demonstrates that women in the follicular phase of menstrual cycle are less responsive to kisspeptin-10 administration than men.

Women in the preovulatory phase of the menstrual cycle are known to be significantly more sensitive to the effects of kisspeptin-54 on gonadotrophin release when compared with
women in the follicular phase of the menstrual cycle (Dhillo et al. 2007). In keeping with this observation, I found an intravenous bolus injection of kisspeptin-10 significantly stimulated LH and FSH release in women during the preovulatory phase of the menstrual cycle.

The pharmacokinetic profiles of plasma kisspeptin IR in both the follicular and preovulatory phases were similar; these results suggest that as with kisspeptin-54, women have heightened sensitivity to kisspeptin-10 during the preovulatory phase of the menstrual cycle.

A recently published study also demonstrated a difference in response to kisspeptin-10 across the menstrual cycle, with stimulation in gonadotrophin release in the luteal and preovulatory phase and an inconsistent response seen in the follicular phase (Chan et al. 2012). The authors postulate that GnRH neurones may have varied responsiveness to exogenous kisspeptin stimulation across the menstrual cycle.

Studies in rodents have identified two distinct kisspeptin neuronal populations that have different responses to oestradiol. In female rodents, c-fos expression within kisspeptin neurones and levels of KISS1 expression are increased within the AVPV nucleus of the hypothalamus immediately preceding ovulation as a result of positive feedback on this nucleus by oestradiol (Smith et al. 2006). This contrasts against the negative feedback by oestradiol on kisspeptin neurones in the ARC, as evidenced by reduced levels of ARC KISS1r expression in rat hypothalamic fragments at proestrus when compared with diestrus (Roa et al. 2006).

In humans KISS1 expressing neurones have only been identified in the infundibular nucleus and to a lesser extent the medial preoptic area (Rometo et al. 2007). In the low oestrogen state of menopause there is significant increase in KISS1 expression in the infundibular nucleus together with hypertrophy and increased numbers of KISS1-expressing neurones when compared to pre-menopausal specimens (Rance 2009).

Guerriero et al. reported that in female rhesus monkeys low doses of kisspeptin-10 stimulated larger GnRH responses in pubertal monkeys compared to prepubertal monkeys.
(Guerriero et al. 2012). Oestradiol levels are higher in pubertal monkeys and ovariectomy in these animals, which reduces oestradiol levels, eliminated the GnRH response to low and high doses of kisspeptin-10. However, the replacement of oestradiol in pubertal monkeys who had undergone ovariectomy resulted in only a partial restoration of the GnRH response to kisspeptin-10; suggesting an effect of another ovarian factor in addition to oestradio on the response to kisspeptin (Guerriero et al. 2012).

In summary, in the course of this thesis I have reported on the first studies examining the effects of kisspeptin-54 in a model of infertility due to hypothalamic amenorrhoea and I have conducted the first clinical study to compare the effects of kisspeptin-10 administration on reproductive hormone release in healthy men and women. These studies suggest several important implications for the potential therapeutic use of kisspeptin-10 or kisspeptin-54 to treat disorders of reproduction.

My future work will investigate the effects of kisspeptin to improve in vitro fertilisation (IVF) treatment for infertility. Infertility is commonly defined as the inability to conceive after two years of regular unprotected sexual intercourse (HFEA Fertility Facts and Figures 2008). Infertility has a high prevalence; it is estimated that around one in six UK couples have difficulty conceiving i.e. approximately 3.5 million couples (HFEA Fertility Facts and Figures 2008). The inability to have children can be devastating, and has important implications for mental, social, and reproductive health. IVF treatment is widely and successfully used to allow infertile couples to conceive and is now approved by the National Institute for Health and Clinical Excellence. However, the most common serious complication of IVF is ovarian hyperstimulation syndrome (OHSS). This is a potentially life threatening condition which has significant morbidity and mortality (RCOG guideline 5, 2006). The major cause of OHSS is the use of human chorionic gonadotrophin (hCG) in current IVF protocols for oocyte maturation (Golan et al. 1989). Human chorionic gonadotrophin binds to the LH receptor and therefore mimics the actions of endogenous LH. However, hCG has a much longer circulating half life than LH; this results in overstimulation of the corpus luteum which is the
underlying cause of OHSS (Damewood et al. 1989). Human chorionic gonadotrophin circulates for up to a week after injection (Fauser et al. 2002), whereas the physiological stimulus, the LH surge, only lasts for 48 hours (Hoff et al. 1983). This overstimulation of the corpus luteum also results in an environment more hostile to embryo implantation (Macklon et al. 2006). A more physiological method for oocyte maturation in IVF treatment should prevent overstimulation of the corpus luteum and thus the subsequent development of OHSS (Macklon et al. 2006). Kisspeptin offers a novel approach in IVF therapy to improve pregnancy rates and avoid the complication of OHSS. Since kisspeptin stimulates the release of physiological levels of GnRH and the consequent release of endogenous gonadotrophins (Irwig et al. 2004; Thompson et al. 2004; Messager et al. 2005) it should lead to a physiological LH surge. A more physiological approach for oocyte maturation in IVF treatment would prevent overstimulation of the corpus luteum and OHSS in IVF treatment (Macklon et al. 2006). The significant advantage of kisspeptin over current treatments is that its effects would depend on the sensitivity of an individual’s hypothalamic-pituitary gonadal (HPG) axis. This would result in a more physiological LH surge and oocyte maturation during IVF treatment, thereby improving safety and efficacy of IVF treatment.

I am planning a future study in which I hypothesise that the use of kisspeptin in place of hCG in IVF protocols will result in the physiological release of the endogenous releasable pool of GnRH and subsequent gonadotrophin secretion; this in turn will lead to oocyte maturation. The aim of this study will be to provide proof of concept that administration of kisspeptin can induce oocyte maturation. Thus kisspeptin may have an exciting role in the treatment of infertility in the future.
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Appendix 1 Principle of radioimmunoassay

Radioimmunoasasy (RIA) is a technique in which unlabelled and radioactively labelled antigen (peptide) compete for an antibody binding site. Specificity is dependent upon the ability of the antibody to recognise subtle structural features of the antigen molecule. The most commonly used radiolabel is I-125, and antibodies are often obtained by immunising rabbits with antigen (peptide) plus adjuvant (though polyclonal antibodies from other species and monoclonal antibodies can be used). In the RIA both the labelled peptide and antibody are at specific, constant concentrations. The concentration of antibody is limiting, so antigen binding will be finite. This allows determination of the amount of unlabelled peptide in a sample. Unlabelled peptide in a sample will compete with labelled peptide for antibody binding. Thus the amount of radiolabelled antigen bound to the antibody is inversely proportional to the amount of unlabelled antigen in the sample being assayed. A ‘standard curve’ is determined using known concentrations of pure peptide to calculate the percentage of labelled peptide bound for each concentration of unlabelled pure peptide. Separation of the unbound radiolabelled antigen from the radiolabelled antigen-antibody complex and counting the proportion of radiolabel present in the two fractions enables direct measurement of the amount of unlabelled antigen that has bound to the antibody. By reference to the standard curve, the unknown concentrations of peptide in the sample can be obtained by interpolation.

Schematic representation of the principle of RIA:

\[ \text{Ag}^* + \text{Ab} + \text{Ag} \leftrightarrow \text{Ag}^*\text{Ab} + \text{AgAb} \]

- \(\text{Ag}^*\) = radiolabelled antigen
- \(\text{Ag}\) = unlabelled antigen
- \(\text{Ab}\) = antibody
Various techniques may be used in order to distinguish antibody-bound antigen from free antigen, and to determine the distribution of radioactive antigen between the two fractions. The methods used in this work are separation by adsorption with charcoal (free radiolabelled antigen is contained in the charcoal pellet following centrifugation), or using a primary antibody / secondary antibody complex (free label is contained in the supernatant following centrifugation).

With charcoal separation, dextran is added to a charcoal suspension to block the larger holes in porous charcoal. The suspension is then added to the RIA tubes, where it traps the free (unbound) radiolabelled antigen. The tubes are then centrifuged and the supernatant (containing antibody-antigen complex) and charcoal pellet (free radiolabelled antigen) are separated by aspiration with a Pasteur pipette. The bound and free labels are counted in a gamma counter. With the secondary antibody separation method, the secondary antibody is derived from an animal species different from the species used to generate primary (antigen-binding) antibody. For example, the leptin primary antiserum is raised in a rabbit, and separation is achieved by using a goat anti-rabbit secondary antibody. Free radiolabelled antigen is in the supernatant, and the pellet contains antigen-antibody complexes. As with charcoal separation, bound and free label are counted in a gamma counter after incubation, centrifugation and separation.

Each RIA has optimum reaction conditions with respect to buffer medium, assay volume, antibody titre, incubation time, temperature, and separation method used. Phosphate buffers are used for a wide range of RIAs. The in-house departmental assays used here are incubated for 3-5 days.

All samples are added in duplicate. “Non-specific binding” tubes, minus primary antibody, are added at the beginning of the assay. Tubes containing half and twice the standard
volume of added labelled antigen are also included in order to confirm the quality of the label. Tubes with no samples (‘zero’ tubes) are placed at regular intervals throughout the assay, and standard curves (containing known quantities of unlabelled antigen) are performed at the beginning and end of each assay, in order to calibrate the assay. The analytical sensitivity of an assay is determined by the smallest change in hormone concentration that can be reliably detected. This is essentially governed by the steepness of the standard curve, and the error of a known value on the curve. The analytical sensitivity is calculated as two standard deviations from the mean concentration of antigen measured in the zero standards (which contains no unlabelled peptide)

**Kisspeptin radioimmunoassay:**

Measurement of plasma kisspeptin immunoreactivity (IR) was performed using an established radioimmunoassay (RIA) (Dhillo et al. 2005; Dhillo et al. 2007). A rabbit antiserum was raised against kisspeptin. The antibody cross-reacted 100% with human kisspeptin-54, kisspeptin-14, and kisspeptin-10 and less than 0.01% with other related RF amide proteins, including prolactin-releasing peptide, RF amide-related peptide 1 (RFRP1), RFRP2, RFRP3, QRFP43, neuropeptide FF, and neuropeptide AF. The limit of detectability was 2pmol/l, and the intra- and interassay coefficients of variation were 8.3 and 10.2%, respectively.

All kisspeptin RIAs were performed using 0.06M phosphate buffer (0.05M Na₂HPO₄·2H₂O, 0.006M KH₂PO₄, 0.01M disodium-EDTA·2H₂O, 0.008M NaN₃) (pH 7.4). A standard concentration of 0.5 pmol/mL kisspeptin-54 was used. Radiolabelled kisspeptin with a radioactivity 25-30 Bq/mL was used. Antiserum was used at a final dilution of 1:3,000,000. Assay tubes were set up in duplicate according to the volumes described in Table 10. Assays were incubated for 3-5 days at 4°C then separated using charcoal adsorption.
Table 10. Volumes of reactants for addition to kisspeptin radioimmunoassay.

NSB, non-specific binding; 1/2 X, addition of half-volume of labelled kisspeptin; 2 X, addition of double volume of labelled kisspeptin; XS, addition of excess volume of antibody.

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<th>LABEL VOLUME (µL)</th>
<th>ANTIBODY VOLUME (µL)</th>
<th>SAMPLE STANDARD / TOTAL VOLUME (µL)</th>
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178
Appendix 2 Original Publications
Subcutaneous Injection of Kisspeptin-54 Acutely Stimulates Gonadotropin Secretion in Women with Hypothalamic Amenorrhea, But Chronic Administration Causes Tachyphylaxis

Channa N. Jayasena, Gurjinder M. K. Nijher, Owais B. Chaudhri, Kevin G. Murphy, Amita Ranger, Adrian Lim, Daksha Patel, Amrish Mehta, Catriona Todd, Radha Ramachandran, Victoria Salem, Gordon W. Stamp, Mandy Donaldson, Mohammad A. Ghaedi, Stephen R. Bloom, and Waljit S. Dhill

Department of Investigative Medicine (C.N.J., G.M.K.N., O.B.C., K.G.M., A.R., R.R., V.S., M.A.G., S.R.B., W.S.D.), Imperial College London, Hammersmith Hospital, London W12 ONN, United Kingdom; Imaging Department (A.L., D.P., A.M., C.T.), Imperial College Healthcare NHS Trust, Charing Cross Hospital, London W6 8RF, United Kingdom; Department of Histopathology (G.W.S.), Imperial College London, Hammersmith Hospital, London W12 ONN, United Kingdom; and Department of Clinical Biochemistry (M.D.), Imperial College Healthcare NHS Trust, Charing Cross Hospital, London W6 8RF, United Kingdom

Background: Kisspeptin is a critical regulator of normal reproductive function. A single injection of kisspeptin in healthy human volunteers potently stimulates gonadotropin release. However, the effects of kisspeptin on gonadotropin release in women with hypothalamic amenorrhea (HA) and the effects of repeated administration of kisspeptin to humans are unknown.

Aim: The aim of this study was to determine the effects of acute and chronic kisspeptin administration on gonadotropin release in women with HA.

Methods: We performed a prospective, randomized, double-blinded, parallel design study. Women with HA received twice-daily sc injections of kisspeptin (6.4 nmol/kg) or 0.9% saline (n = 110 per group) for 2 wk. Changes in serum gonadotropin and estradiol levels, LH pulsatility, and ultrasound measurements of reproductive activity were assessed.

Results: On the first injection day, potent increases in serum LH and FSH were observed after sc kisspeptin injection in women with HA (mean maximal increment from baseline within 4 h after injection: LH, 24.0 ± 3.5 IU/liter; FSH, 9.1 ± 2.5 IU/liter). These responses were significantly reduced on the 14th injection day (mean maximal increment from baseline within 4 h postinjection: LH, 2.5 ± 2.2 IU/liter, P < 0.05; FSH, 0.5 ± 0.5 IU/liter, P < 0.05). Subjects remained responsive to GnRH after kisspeptin treatment. No significant changes in LH pulsatility or ultrasound measurements of reproductive activity were observed.

Conclusion: Acute administration of kisspeptin to women with infertility due to HA potently stimulates gonadotropin release, but chronic administration of kisspeptin results in desensitization to its effects on gonadotropin release. These data have important implications for the development of kisspeptin as a novel therapy for reproductive disorders in humans. (J Clin Endocrinol Metab 94: 4315–4323, 2009)
Hypothalamic amenorrhea (HA) is defined as the cessation of menstruation due to abnormal signaling between the hypothalamus and the pituitary gland (1), and it accounts for approximately 30% of cases of amenorrhea in women of reproductive age (2). Functional HA is defined as HA occurring in the absence of a structural lesion and often results from a relative energy deficit within the body (low body weight or weight loss) (3–7). Although current treatments for women with HA such as clomiphene, gonadotropin injections, and GnRH pump therapy are efficacious, each has associated limitations (8, 9).

The kisspeptins are a group of arginine-phenylalanine (RF) amide peptides, encoded by the KISS1 gene, that have been identified as potential novel agents for treating reproductive disorders. They act as endogenous ligands for the kisspeptin receptor (KISS1R, alternatively known as G protein-coupled receptor 54) (10–12). KISS1 and KISS1R are expressed in the hypothalamus, pituitary, and placenta (10, 12–14). Kisspeptin signaling exerts powerful effects on the mammalian reproductive system. Mice lacking kisspeptin or the kisspeptin receptor fail to undergo puberty and are infertile (15, 16). In humans, inactivating mutations of KISS1R cause pubertal failure (16, 17), and activating mutations lead to precocious puberty (18). Furthermore, central or peripheral administration of kisspeptin induces gonadotropin and sex steroid release in all mammalian species investigated, including rats (19–21), mice (22, 23), monkeys (24) and sheep (23, 25). We have previously demonstrated that iv infusion or sc bolus injection of kisspeptin-54 stimulates gonadotropin secretion in healthy human male and female subjects, respectively (26, 27). Kisspeptin may therefore be a potential novel therapy for treating reproductive disorders in humans. However, the effects of kisspeptin administration in patients with infertility have not been previously investigated.

Although it has been consistently demonstrated that acute administration of kisspeptin stimulates gonadotropin release (19–27), the effects of chronic administration of kisspeptin on reproductive function are less clear. Chronic administration of kisspeptin to nonhuman mammals causes either sustained or nonsustained stimulation of reproductive function, depending on the mode of administration used. Intermittent administration of kisspeptin-10 to juvenile female rats (twice-daily injections) for 5 d and juvenile male monkeys (hourly injections) for 2 d induces precocious reproductive maturation (28, 29). In contrast, continuous peripheral infusion of kisspeptin-10 to monkeys or rats increases LH release only during the first 3 h or the first day of administration, respectively; LH concentrations subsequently return to levels observed before infusion of kisspeptin-10 (30, 31). The long-term effects of administration of kisspeptin in humans have not been studied to date.

In prepubertal female rats, caloric restriction leads to reduced gonadotropin levels, delayed vaginal opening, and low hypothalamic kiss1 expression (32). Twice-daily administration of kisspeptin-10 to these animals restores vaginal opening and gonadotropin secretion (32). Based on these data, we hypothesized that repetitive administration of kisspeptin would restore gonadotropin secretion in human female subjects with HA.

A randomized, double-blinded, placebo-controlled, parallel design study was conducted to determine whether twice-daily administration of kisspeptin to human female subjects with HA would sustainably stimulate gonadotropin release.

Subjects and Methods

Kisspeptin-54

Kisspeptin-54 was synthesized by the Advanced Biotechnology Centre, Imperial College London, and purified by reverse-phase HPLC. Electrospray mass spectroscopy and amino acid analysis confirmed identity of the peptide as previously described (26, 27). The peptide was tested for bioactivity and toxicity as previously described (26). The Limulus amebocyte lysate assay (Associates of Cape Cod, Liverpool, UK) was negative for endotoxin, and the peptide was sterile on culture (Department of Microbiology, Hammersmith Hospital, London). Although kisspeptin-10, -13, -14, and -54 display similar potency in vitro, we used kisspeptin-54 due to its higher in vivo potency than the other kisspeptin fragments (31, 33).

Subjects

Ethical approval was granted by the Hammersmith and Queen Charlotte’s and Chelsea Hospitals Research Ethics Committee (registration number: 05/Q0406/142). Written informed consent was obtained from all subjects. This study was performed in accordance with the Declaration of Helsinki.

Subjects were recruited through advertisements placed in local newspapers. Responders to advertisements were evaluated with a detailed menstrual history, clinical examination, electrocardiogram, and blood tests. Screening blood tests performed were as follows: full blood count, renal profile, liver profile, bone profile, glucose, thyroid profile, LH, FSH, estradiol, progesterone, androstenedione, dehydroepiandrosterone, testosterone, SHBG, prolactin, 17-hydroxyprogesterone, and cortisol. Women were diagnosed with functional HA and included within the study if they fulfilled the following criteria: body mass index below 25 kg/m²; stable body weight over the previous 6 months; age between 18 and 40 yr; secondary amenorrhea of at least 6 months duration; absence of oral contraceptive pill therapy for at least 1 yr; absence of systemic disease comorbidity; absence of active psychiatric illness; stable body weight; absence of therapeutic or recreational drug use; absence of clinical or biochemical hyperandrogenemia; structurally normal hypothalamic-pituitary region assessed by magnetic resonance imaging; structurally normal female reproductive tract visualized on ultrasound; absence
TABLE 1. Comparison of baseline characteristics of women with HA randomized to saline vs. kisspeptin-54

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Study group</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saline</td>
<td>Kisspeptin-54</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>24.8 ± 0.5</td>
<td>26.8 ± 2.4</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>51.8 ± 3.3</td>
<td>54.5 ± 1.1</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>19.0 ± 0.7</td>
<td>19.9 ± 0.4</td>
</tr>
<tr>
<td>Duration of amenorrhea (months)</td>
<td>22.4 ± 9.9</td>
<td>23.2 ± 12.7</td>
</tr>
<tr>
<td>Serum LH (IU/liter)</td>
<td>4.5 ± 1.6</td>
<td>2.6 ± 0.9</td>
</tr>
<tr>
<td>Serum FSH (IU/liter)</td>
<td>6.6 ± 0.7</td>
<td>6.1 ± 1.0</td>
</tr>
<tr>
<td>Serum estradiol (pmol/liter)</td>
<td>105 ± 13.9</td>
<td>78 ± 4.9</td>
</tr>
</tbody>
</table>

Values are provided for subjects randomized to receive saline (n = 5) or kisspeptin-54 (n = 5). Data are shown as mean ± sem.

Kisspeptin injections
All subjects were trained in self-administration of sc injections by an investigator at the start of the study protocol. At the beginning of each week when injections were to be performed, a box containing unlabeled vials of freeze-dried saline or kisspeptin-54, alcohol wipes, saline vials, needles, and needle disposal bins was given to each subject. For injection, vial contents were reconstituted in 0.5 ml of 0.9% saline. Then a 0.5-ml insulin syringe was used to inject a weight-adjusted volume of dissolved vial contents into the lower anterior abdominal region. Subjects were instructed to refrigerate vials stored at home.

4-h blood sampling after injection of saline or kisspeptin
All subjects underwent blood sampling in the 4-h period immediately after the first (wk 5, d 1) and final (wk 6, d 7) injection of saline (five subjects) or kisspeptin-54 (five subjects) of treatment period. These studies were done in an investigation unit. An unused vial returned by each subject from home storage was used for their final injection of kisspeptin or saline. Saline or kisspeptin-54 (6.4 nmol/kg) was sc administered at 0 min by the investigator, and blood was sampled for serum LH, FSH, estradiol, and SHBG, and plasma kisspeptin-immunoreactivity (IR) at −30, 0, 15, 30, 45, 60, 75, 90, 120, 150, 180, 210, and 240 min. In one subject, the study was extended to include blood sampling at 270, 300, 330, 360, 390, 420, 450, and 480 min postinjection.

Assessments of LH pulsatility
Subjects underwent assessment of LH pulsatility on the first study day (wk 1, d 1) and approximately 24 h after the final injection of the treatment period (wk 7, d 1). Blood was sampled sequentially every 10 min for serum LH over an 8-h period. These studies were commenced between the hours of 0800 h and 1200 h.

Basal measurement of reproductive hormones
Twice-weekly basal measurements of serum LH, FSH, estradiol, progesterone, SHBG, and plasma kisspeptin-IR were taken from subjects throughout the 8-wk study protocol between 0800 and 1800 h. During weeks when injections were self-administered by volunteers (wk 1, 2, 5, and 6), these blood tests were performed a mean of 4.5 ± 0.4 h after the previous injection, depending on the availability of volunteers. These twice-weekly basal measurements were used to calculate mean values for serum LH, FSH, and estradiol during the baseline period (wk 1–4), treatment period (wk 5–6) and posttreatment period (wk 7–8) of the study protocol. Kisspeptin-IR was measured to confirm subject compliance to kisspeptin injections.

Ultrasound scans
Transabdominal ultrasound scans were performed once a week throughout the 8-wk study period. During each scan, the following parameters were measured: endometrial thickness in millimeters; mean ovarian volume in cubic centimeters; mean follicles number; and maximum diameter of largest follicle in each ovary in millimeters. Ovulation was confirmed by satisfaction of all of the following criteria: visualization of a dominant follicle (diameter, 11 mm or greater); enlargement of dominant follicle into a preovulatory follicle (diameter, 18 mm or greater); subsequent collapse of preovulatory follicle or appearance of
internal echoes on ultrasonography; and a rise in serum progesterone to over 10 nmol/liter.

**Other measurements**

Weight was measured on the first study day (wk 1, d 1) and subsequently every 2 wk during the 8-wk protocol. During each study visit, urine was tested to exclude pregnancy (Clearview easy-HCG; Inverness Medical Innovations Inc., Waltham, MA).

Diastolic and systolic blood pressure and heart rate were recorded every 30 min during the LH pulsatility studies, to compare mean values for each parameter before and after the treatment period (wk 5–6). Blood pressure and heart rate were also recorded during 4-h blood sampling studies performed after injection of kisspeptin or saline.

**Response of HA subjects to GnRH before and after injections of kisspeptin**

A second group of five female subjects was recruited using identical inclusion criteria for HA described in this study, to determine whether sensitivity to the effects of GnRH was retained after desensitization to the effects of kisspeptin. A baseline GnRH test was performed in all subjects in the investigation unit. In brief, subjects were cannulated and given a 100-µg iv bolus injection of GnRH (HRF; Intrapharm Ltd., Kent, UK) at 0 min. Blood was sampled for measurement of serum LH, FSH, and estradiol at −30, 0, 15, 30, 45, 60, 90, and 120 min. Seven days after the GnRH test, all five women self-administered twice-daily kisspeptin injections (6.4 nmol/kg) for 2 wk. The GnRH test was repeated in each subject 8–12 h after their final kisspeptin injection.

**Collection and processing of blood samples**

Blood samples for serum analysis were collected in plain serum Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ). Samples were allowed to clot before centrifugation and separation of serum. Blood samples for plasma kisspeptin analysis were collected in lithium heparin tubes (Becton Dickinson) containing 5000 kallikrein inhibitor units of aprotinin (0.2 ml Trasylol; Bayer, Newbury, UK). Samples were immediately centrifuged at room temperature using a Hettich EBA 20 machine (Hettich International, Tuttinglen, Germany) for 10 min at 3000 rpm and then separated. Serum and plasma samples were stored at −20°C until analysis.

**Analytical methods**

Serum LH, FSH, estradiol, and progesterone were measured using automated chemiluminescent immunoassays (Abbott Diagnostics, Maidenhead, UK). SHBG was measured using a solid-phase automated enzyme immunoassay (Immulite; Sievens, Llanberis, UK). Reference ranges for females were as follows: LH (follicular), 2–10 IU/liter; LH (midcycle), 20–60 IU/liter; LH (luteal), 4–14 IU/liter; FSH (follicular and luteal), 1.5–8 IU/liter; estradiol (early follicular), less than 300 pmol/liter; estradiol (midcycle), 400–1500 pmol/liter; estradiol (luteal), 200–1000 pmol/liter; and SHBG, 40–80 nmol/liter. Interassay coefficients of variation were as follows: LH, 3.4%; FSH, 3.5%; estradiol, 3.4%; progesterone, 1.8%; and SHBG, 5.6%. Limits of detectability for each assay were as follows: estradiol, 70 pmol/liter; FSH, 0.05 mlU/ml; LH, 0.07 mlU/ml; progesterone, 0.1 ng/ml; and SHBG, 0.1 nmol/liter.

Measurement of plasma kisspeptin immunoreactivity (IR) was performed using an established RIA (26, 27). The antibody cross-reacted 100% with human kisspeptin-54, kisspeptin-14, and kisspeptin-10 and less than 0.01% with other related RF amide proteins, including prolactin-releasing peptide, RF amide-related peptide 1 (RFRP1), RFRP2, RFRP3, QRFP 43, neuropeptide FF, and neuropeptide AF. The limit of detectability was 2 pmol/liter, and the intra- and interassay coefficients of variation were 8.3 and 10.2%, respectively.

**Data analysis**

Data are presented as mean ± sem. Hormone profiles during 4-h blood sampling studies and GnRH tests were analyzed using repeated measures two-way ANOVA with Bonferroni post hoc correction. Pairs of means were analyzed using the unpaired two-tailed t test. Multiple means were compared using one-way ANOVA with Bonferroni’s Multiple Comparison Test. A previously described modified Santen and Bardin method was used to assess LH pulsatility (34, 35). In all cases, P < 0.05 was considered statistically significant.

**Results**

**Characteristics of subjects recruited to the study**

Baseline age, weight, and body mass index were not significantly different between kisspeptin and saline study groups (Table 1). Weight remained stable in both treatment groups during the study (mean weight change from beginning to end of study: saline, −0.5 kg; kisspeptin, −0.1 kg; P = 0.09). Subjects reported no increased incidence of nausea or other side effects after injection of kisspeptin or saline. Mean heart rate and systolic and diastolic blood pressure were similar before and after the treatment period (wk 5–6) in all participants (data not shown). Furthermore, no significant acute changes in heart rate or systolic and diastolic blood pressure were observed after kisspeptin administration when compared with saline control (data not shown).

**Kisspeptin immunoreactivity in plasma was raised after injection of kisspeptin**

Baseline plasma kisspeptin-IR was below 2 pmol/liter and remained unchanged during the 4-h period after injection of saline (Fig. 1, A and B). Kisspeptin injection resulted in a rise in plasma kisspeptin-IR, with peak mean kisspeptin-IR of approximately 5000 pmol/liter at 45 min after injection (Fig. 1, A and B). Similar patterns of kisspeptin-IR were observed after injection of kisspeptin on the first and last injection days (Fig. 1, A and B).

In subjects randomized to receive saline injections, plasma kisspeptin-IR remained less than 2 pmol/liter during basal measurements taken throughout the 8-wk study protocol (Fig. 1D). In subjects randomized to receive kisspeptin injections, plasma kisspeptin-IR was raised...
during the 2-wk kisspeptin treatment period (wk 5–6) but remained below 2 pmol/liter for the remainder of the study protocol (Fig. 1C). These twice-weekly blood samples were taken at various times of the day (determined by subject availability) between the twice-daily injections. Accordingly, the mean kisspeptin-IR values during the treatment period (wk 5, mean plasma kisspeptin-IR, 416 ± 217 pmol/liter; and wk 6, mean plasma kisspeptin-IR, 751 ± 252 pmol/liter) were lower than the peak kisspeptin-IR observed 45 min after kisspeptin injection.

Effects of first injection of saline or kisspeptin on serum reproductive hormones in women with HA

On the first injection day of the treatment period (wk 5, d 1), saline injection did not change serum LH, FSH, or estradiol levels compared with baseline (Fig. 2, A–C). Kisspeptin-54 injection acutely and potently increased serum LH levels in subjects with HA in comparison to saline ($P < 0.001$ at time points 150 to 240 min; Fig. 2A). The mean maximal increase in LH from baseline after kisspeptin injection was observed at 240 min and was $24.0 \pm 3.5$ IU/liter above baseline. Kisspeptin-54 injection also potently increased serum FSH levels compared with saline ($P < 0.001$ at time points 180 to 240 min; Fig. 2B). After kisspeptin injection, the maximal FSH rise was observed at 240 min and was $9.1 \pm 2.5$ IU/liter above baseline. Estradiol levels after kisspeptin injection were initially similar to those following saline. However, estradiol levels significantly increased above baseline between 180 and 240 min after kisspeptin administration ($P < 0.05$; Fig. 2C).

Effects of last injection of saline or kisspeptin on serum reproductive hormones in women with HA

On the last injection day of the treatment period (wk 6, d 7), saline injection did not change serum LH, FSH, or estradiol levels compared with baseline (Fig. 2, D–F). Kisspeptin administration resulted in a significant rise in LH only at 240 min after injection and no significant rises in FSH or estradiol. Responses of LH, FSH, and estradiol to the kisspeptin administration were all significantly reduced after the last injection (wk 6, d 7) when compared with the responses after the first kisspeptin injection (wk 5, d 1) ($P < 0.05$ for LH, FSH, and estradiol responses).
FIG. 2. Effects of the first and last injections of saline or kisspeptin-54 on serum reproductive hormones in women with HA. A–C, Changes in serum LH (A), FSH (B), and estradiol (C) after bolus sc injection of saline (n = 5) or 6.4 nmol/kg kisspeptin-54 (KP54, n = 5) on first day (wk 5, d 1) of treatment period are shown. D–F, Changes in serum LH (D), FSH (E), and estradiol (F) after bolus sc injection of saline or kisspeptin-54 on the last day (wk 6, d 7) of the treatment period are shown. Injections were administered at 0 min. Data are shown as mean ± SEM. *, P < 0.05; ***, P < 0.001.

E2, Estradiol.

After the observation of significantly reduced gonadotropin responses to kisspeptin on the last injection day, we decided to assess further the duration of response to kisspeptin injection in one volunteer in whom blood sampling was extended to 8 h after injection. In this volunteer, kisspeptin-IR was raised until 6 h after injection (data not shown). Furthermore, serum LH, FSH, and estradiol were still raised above baseline by the end of the 8-h sampling period (data not shown).

We also examined responsiveness to iv GnRH in five additional subjects with HA, both before and after kisspeptin treatment. We observed LH responses to GnRH in all subjects before commencing kisspeptin treatment (mean peak LH increase during first 2 h after GnRH injection, 14.4 ± 4.6 IU/liter) (Fig. 3). Furthermore these subjects remained responsive to GnRH injection 8–12 h after their final kisspeptin injection (P = 0.23 vs. baseline LH response, using two-way ANOVA) (Fig. 3).

Reproductive hormones, LH pulsatility pattern, and radiological findings after saline or kisspeptin treatment

Mean LH levels from twice-weekly basal blood tests during wk 1 to 4 (the baseline period) were slightly lower in the kisspeptin group than the saline group [mean LH, saline 3.7 ± 0.6 vs. kisspeptin 1.8 ± 0.3; P < 0.05; Supplementary Table 1, published as supplemental data on The Endocrine Society’s Journals Online web site at http://jcem.endojournals.org]. Small rises in mean basal LH and FSH levels were detected during wk 5 to 6 (the treatment period) in women randomized to kisspeptin vs. saline (mean basal LH, saline −0.8 ± 0.7 vs. kisspeptin +1.2 ± 0.7; P = 0.09; mean basal FSH, saline −1.8 ± 0.4 vs. kisspeptin +0.6 ± 0.4; P < 0.05). Basal serum reproduc-

FIG. 3. Comparison of LH responses to GnRH administration before and after kisspeptin-54 injections in women with HA. Intravenous GnRH (100 µg) was administered 7 d before commencement of a 14-d, twice-daily regime of sc kisspeptin injections (6.4 nmol/kg) (baseline response to GnRH) (n = 5). The GnRH test was repeated 8–12 h after the last injection of kisspeptin (posttreatment response to GnRH). When comparing baseline and posttreatment LH responses to GnRH injection, overall responses were similar (P = 0.23), as were LH changes at each time-point after GnRH injection. Injections were administered at 0 min. Data are shown as mean ± SEM.
tive hormone levels were otherwise similar between kisspeptin and saline groups throughout the 8-wk study period.

There was no significant change in mean LH, number of LH pulses, or mean pulse amplitude observed in patients receiving saline or kisspeptin (Supplementary Table 2).

There was no significant change in mean values for endometrial thickness, ovarian volume, follicle number, or maximum follicle diameter observed after kisspeptin vs. saline treatment (Supplementary Table 3). One subject receiving kisspeptin developed radiological changes suggesting possible ovulation, with subsequent symptoms of premenstruation. Ultrasound scans of the subject revealed the rupture of a preovulatory follicle (19-mm diameter) together with subsequent appearance of a corpus luteum. However, this subject had no detectable rise in serum progesterone level and reported no menstrual bleeding.

Discussion

We report the first study of kisspeptin administration in a human model of infertility and the first investigation of the effects of chronic administration of kisspeptin in humans. Our results show that acute administration of kisspeptin-54 increased serum gonadotropin levels in women with HA but repeated injections lead to reduced effect.

Subjects with HA display a reproductive hormone profile that resembles the follicular phase of the menstrual cycle (low circulating gonadotropin and estradiol levels) more closely than the other phases. Acute LH response to kisspeptin injection was approximately 4-fold greater in patients with HA than in previously studied healthy females in the follicular phase of the menstrual cycle given an identical weight-adjusted dose (mean area under curve LH increase during first 4 h post-kisspeptin injection in h.iU/liter: women with HA in the current study, 40.2; healthy females in follicular phase, 9.8; P < 0.01) (27). This is consistent with the observation that LH responses to kisspeptin-10 may be higher in undenourished female rats when compared with those fed ad libitum (32). Further work in a single study comparing women with HA and women with normal menstrual cycles is required to confirm the observation made in this study. If confirmed, it would be interesting to determine whether increased responsiveness of women with HA to kisspeptin is attributable to factors such as increased sensitivity to kisspeptin itself or increased pituitary sensitivity to GnRH.

In juvenile female rats, twice-daily intracerebroventricular administration of kisspeptin-10 induces precocious vaginal opening in ad libitum fed animals (28) and restores vaginal opening under conditions of caloric restriction (32). Furthermore, Plant et al. (29) found that hourly iv kisspeptin-10 pulses were sufficient to induce a train of GnRH discharges characteristic of puberty in juvenile monkeys. We were therefore surprised to observe that LH, FSH, and estradiol responses to the last kisspeptin injection were markedly lower than responses to the first injection. In addition, reproductive ultrasound and basal reproductive hormone parameters were similar between the two treatment groups. The last kisspeptin injection, which was reconstituted using peptide returned from home storage by each volunteer, led to similarly elevated plasma kisspeptin-IR to that observed after the first injection. This suggests that peptide degradation caused by home storage of kisspeptin did not account for the markedly reduced gonadotropin responses to kisspeptin on the last injection day.

Kisspeptin-10 was used during the animal studies of repetitive kisspeptin administration (28, 29), whereas the 54-amino acid form of kisspeptin was administered during this study. Our results reveal that plasma kisspeptin-IR is raised for up to 6 h after each sc injection of kisspeptin-54. Sustained exposure of monkeys and rodents to kisspeptin also leads to desensitization to its effects. Seminara et al. (30) demonstrated that continuous iv kisspeptin-10 administration to male rhesus monkeys for 98 h led to increased LH release lasting only 3 h, followed by a return of gonadotropin concentrations to levels similar to those observed before the kisspeptin-10 infusion. Similarly, Thompson et al. (31) observed increased LH levels only during the first day of a continuous 3-d sc kisspeptin-54 infusion to adult male rats. A recent publication by Keen et al. (36) demonstrates the pattern of kisspeptin release within the monkey hypothalamic median eminence to be pulsatile. Animal data suggest that a protocol using intermittent administration of kisspeptin (28–29, 32) may be less likely to result in desensitization than a protocol using continuous administration (30, 31). However, given the prolonged action of sc kisspeptin-54 injection on plasma kisspeptin-IR and reproductive hormone levels, our protocol of twice-daily kisspeptin-54 injections may have resulted in desensitization through prolonged and nonpulsatile kisspeptin exposure. An intermittent, iv method of kisspeptin administration might minimize or prevent the desensitization of gonadotropin responses observed in this study.

We observed GnRH administration to stimulate LH secretion in HA subjects even after 2 wk of kisspeptin treatment. A study by Ramaswamy et al. (37) demonstrated that responsiveness to GnRH bolus was maintained in adult male rhesus monkeys during an infusion of kisspeptin-10 delivered at 200 µg/h but was reduced at a higher infusion rate of 400 µg/h. In our study, a lower LH response to GnRH administration was observed after
kisspeptin treatment when compared with the baseline response; however, this difference was not significant. Our results therefore suggest that the protocol of kisspeptin administration used during this study led to desensitization upstream of the pituitary gland. It is possible that our observations are explained by KISS1R down-regulation, which has been previously demonstrated in vitro (38).

Kisspeptin antagonist has been shown to inhibit pulsatile GnRH release in pubertal female rhesus monkeys and pulsatile LH release in adult female sheep (39). Furthermore, Ramaswamy et al. (37) observed that LH pulse amplitude and frequency was reduced by infusion of kisspeptin-10 at 400 µg/h (but not 200 µg/h) in adult male monkeys. In the current study, neither LH pulse amplitude nor LH pulse frequency was significantly altered after kisspeptin treatment in women with HA. Our results might be explained by rapid recovery from kisspeptin exposure during the 24-h period between the final kisspeptin injection and the second assessment of LH pulsatility study. It is also plausible that a protocol using higher or more frequent doses of kisspeptin injections would have significantly altered LH pulsatility.

This study demonstrates that acute sc administration of kisspeptin-54 potently stimulates pituitary-gonadal function in human females with HA. However, significantly reduced gonadotropin responses to kisspeptin-54 administration were observed after 2 wk of twice-daily kisspeptin-54 injections, suggesting desensitization. These results have important implications for the therapeutic potential of kisspeptin to treat patients with reproductive disorders.

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Twice-Weekly Administration of Kisspeptin-54 for 8 Weeks Stimulates Release of Reproductive Hormones in Women With Hypothalamic Amenorrhea

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Kisspeptin is a novel therapeutic target for infertility. A single kisspeptin-54 (KP-54) injection acutely stimulates the release of reproductive hormones in women with hypothalamic amenorrhea (HA), a commonly occurring condition characterized by absence of menstruation; however, twice-daily administration of KP-54 results in tachyphylaxis. We determined the time course of desensitization to twice-daily KP-54 injections, compared the effects of twice-daily and twice-weekly administration regimens of KP-54, and studied the effects of long-term twice-weekly administration of KP-54 on the release of reproductive hormones in women with HA. When KP-54 was administered twice daily, responsiveness to luteinizing hormone (LH) diminished gradually, whereas responsiveness to follicle-stimulating hormone (FSH) was nearly abolished by day 2. Twice-weekly KP-54 administration resulted in only partial desensitization, in contrast to the complete tolerance achieved with twice-daily administration. Women with HA who were treated with twice-weekly KP-54 injections had significantly elevated levels of reproductive hormones after 8 weeks as compared with treatment with saline. No adverse effects were observed. This study provides novel pharmacological data on the effects of KP-54 on the release of reproductive hormones in women with HA.

Infertility affects 9% of couples worldwide.1 Gonadotrophin injections containing luteinizing hormone (LH) or follicle-stimulating hormone (FSH) are routinely used to treat such patients. Although this treatment is efficacious, it is associated with the risk of the potentially life-threatening condition known as ovarian hyperstimulation syndrome.2 It has recently emerged that kisspeptin plays a critical role in the mammalian reproductive system and may therefore offer a novel therapeutic target in the treatment of patients with reproductive disorders.

Kisspeptin is a group of arginine–phenylalanine amide peptides encoded by the KISS1 gene, which is expressed in the hypothalamus, pituitary gland, and gonads.3,4 In humans, inactivating mutations of the kisspeptin receptor (KISS1R) cause pubertal failure,5,6 and activating mutations lead to precocious puberty.7 In all the mammalian species that have been investigated, central or peripheral administration of kisspeptin induces the release of gonadotrophin.8–16 This effect is abolished by preadministration of an antagonist to the hypothalamic hormone gonadotrophin-releasing hormone.13 Kisspeptin is therefore thought to indirectly stimulate the release of gonadotrophin from the pituitary gland by stimulating the release of gonadotrophin-releasing hormone from the hypothalamus. We have previously demonstrated that single injections of kisspeptin-54 (KP-54) stimulate the secretion of gonadotrophin in healthy human male and female subjects.15,16 It is therefore of interest to investigate whether KP-54 can be used to stimulate the release of gonadotrophin in patients with infertility conditions that are associated with deficient secretion of gonadotrophin (hypogonadotrophic hypogonadism).

Hypothalamic amenorrhea (HA) is a form of hypogonadotrophic hypogonadism17 that accounts for ~30% of amenorrhea

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Functional HA is defined as occurring in the absence of a structural hypothalamo-pituitary lesion and often results from a low body weight or significant weight loss. We previously demonstrated that a single injection of KP-54 stimulates the release of gonadotrophin in women with HA. However, women with HA who were treated with twice-daily KP-54 injections for 2 weeks were markedly less responsive to KP-54 at the end of the study as compared with the start of the study. Interestingly, these women remained responsive to gonadotrophin-releasing hormone injection at the end of the study, thereby suggesting that the desensitization associated with repeated administration of KP-54 was occurring at the KISS1R. Consequently, acute administration of KP-54 to women with HA results in a potent stimulation of LH release, but twice-daily KP-54 administration for 2 weeks leads to tachyphylaxis.

Determining whether KP-54 can stimulate gonadotrophin release in a sustained manner has important implications for the treatment of patients with infertility. We hypothesized that an alternative protocol of repeated KP-54 administration would sustainably stimulate the release of gonadotrophin in women with HA. The aims of this randomized, double-blinded, placebo-controlled parallel design study were to determine (i) the time course over which desensitization to the effects of KP-54 on the release of reproductive hormone occurs in women with HA, (ii) the effects of prolongation of the KP-54 dose interval on desensitization to KP-54 treatment, and (iii) whether the administration of KP-54 twice weekly can result in chronic stimulation of gonadotrophin release and restore menstrual cyclicity in women with HA over an 8-week period.

RESULTS

Characteristics of subjects recruited to the study
Baseline age, weight, and body mass index were similar for all groups of subjects in this study (Table 1).

Study 1: time course of desensitization to the gonadotrophin-inducing effects of KP-54 in women with HA

Twice-daily subcutaneous administration of 6.4 nmol/kg KP-54 was associated with a progressive reduction in acute LH responses after injection of KP-54 in women with HA (Figure 1a). On day 1, the mean maximal LH response during the first 4 h after KP-54 injection was 23.3 ± 12.1 IU/l; however, the mean maximal LH response during this period dropped to 11.2 ± 5.7, 4.2 ± 1.1, 3.4 ± 0.5, and 1.1 ± 0.5 IU/l on days 2, 3, 4, and 14 of injections, respectively (Figure 1a). On day 1, the mean maximal FSH response during the first 4 h after the KP-54 injection was 7.2 ± 2.2 h·IU/l. However, the acute FSH responses after the injection were <2 U/l on days 2, 3, 4, and 14 (Figure 1b). Mean estradiol levels were not significantly different at any point during the study (Figure 1c).

Study 2: two-week study of release of reproductive hormones in serum with HA receiving twice-daily KP-54 injections as compared with those receiving twice-weekly injections

A. Effect of twice-daily subcutaneous injection of KP-54 (6.4 nmol/kg) on the release of reproductive hormone. Twice-daily subcutaneous injection of 6.4 nmol/kg KP-54 in women with HA potently stimulated gonadotrophin release on day 1, with the maximal increase being observed 240 min after the injection (mean maximal increase following KP-54 injection on day 1: LH: 23.2 ± 11.3 IU/l; FSH: 7.2 ± 2.1 IU/l). However, on day 14, reproductive hormone responses were significantly reduced as compared with those on day 1 (mean maximal increase after KP-54 injection on day 14: LH: 1.0 ± 0.5 IU/l, P < 0.05 relative to day 1; FSH: 0.2 ± 0.3 IU/l, P < 0.001 relative to day 1) (Figure 2a,b). The estradiol response was nonsignificantly lower on day 14 as compared with that at day 1 (mean maximal estradiol increase after the injection: day 1, 48.2 ± 29.7 pmol/l; day 14, −12.7 ± 7.0 pmol/l, P = 0.06) (Figure 2c).

B. Effect of twice-weekly subcutaneous injection of KP-54 (6.4 nmol/kg) on release of reproductive hormone. We had observed that subcutaneous administration of KP-54 twice daily led to significant desensitization of gonadotrophin responses within 2 weeks of treatment. We therefore investigated whether the same subcutaneous dose of KP-54 (6.4 nmol/kg) would be associated

Table 1 Comparison of baseline characteristics of women with hypothalamic amenorrhea participating in the 2-week pilot studies of the effects of administering kisspeptin and the 8-week study of the effects of administering saline vs. those of administering kisspeptin

<table>
<thead>
<tr>
<th>Protocol treatment</th>
<th>2-Week pilot studies</th>
<th>8-Week study</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kisspeptin-54 (n = 10)</td>
<td>Saline (n = 5)</td>
<td>Kisspeptin-54 (n = 5)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>28.1 ± 1.1</td>
<td>27.0 ± 2.6</td>
<td>27.2 ± 2.4</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>52.6 ± 0.7</td>
<td>54.5 ± 3.9</td>
<td>53.4 ± 3.0</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>19.1 ± 0.3</td>
<td>19.7 ± 1.1</td>
<td>19.6 ± 0.7</td>
</tr>
<tr>
<td>Duration of amenorrhea (months)</td>
<td>22.4 ± 9.9</td>
<td>20.4 ± 5.1</td>
<td>28.8 ± 12.0</td>
</tr>
<tr>
<td>Serum LH (IU/l)</td>
<td>2.5 ± 0.6</td>
<td>1.7 ± 0.7</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>Serum FSH (IU/l)</td>
<td>4.8 ± 0.3</td>
<td>3.8 ± 0.9</td>
<td>3.6 ± 0.6</td>
</tr>
<tr>
<td>Serum estradiol (pmol/l)</td>
<td>133 ± 10</td>
<td>87 ± 17</td>
<td>151 ± 28</td>
</tr>
</tbody>
</table>

Mean values ± SEM data are shown.
FSH, follicle-stimulating hormone; LH, luteinizing hormone; NS, nonsignificant difference between the three groups of women with hypothalamic amenorrhea.
with less desensitization if administered twice weekly (rather than twice daily). On day 14, mean gonadotrophin responses after the KP-54 injection were nonsignificantly lower relative to day 1 (mean maximal increase in IU/l after the injection: LH: day 1, 18.8 ± 6.6 vs. day 14, 11.5 ± 4.0, \( P = 0.08 \); FSH: day 1, 5.8 ± 2.0 vs. day 14, 4.1 ± 1.1, \( P = 0.14 \)) (Figures 2d,e). The mean maximal increases in serum estradiol on day 1 after the KP-54 injection was not significantly different from that at day 14 (day 1 44.8 ± 15.7 pmol/l vs. day 14 27.5 ± 15.5 pmol/l; \( P = 0.47 \)) (Figure 2f).

A summary of the effects of subcutaneous administration of KP-54 injections on days 1 and 14 of each of the different 2-week dosing regimens of KP-54 is shown in Figures 2g–i as mean areas under the curve for LH, FSH, and estradiol responses.

**Study 3: effects of twice-weekly KP-54 administration for 8 weeks on reproductive hormone levels in women with HA**

The results from study 2 suggested that twice-weekly administration of KP-54 6.4 nmol/kg significantly reduces desensitization to the effects of KP-54 on the release of reproductive hormone. A randomized, double-blinded, placebo-controlled, parallel design study was therefore performed in order to determine whether twice-weekly administration of KP-54 at 6.4 nmol/kg would stimulate the release of reproductive hormone over an 8-week period (protocol summary shown in Figure 3). Baseline age, weight, and body mass index were not significantly different between the group receiving KP-54 and the control group receiving saline (Table 1). The subjects reported no adverse effects after injections of either KP-54 or saline. No significant acute
changes were observed in heart rate or in systolic or diastolic blood pressure after the administration of KP-54 as compared with saline (data not shown).

Saline had no significant effects on the release of reproductive hormones at any time during the 8-week protocol of twice-weekly injections (Figure 4a–c). As expected, KP-54 stimulated the release of reproductive hormones after the injection on day 1. After 2 weeks of twice-weekly injections, LH responses after the KP-54 injection were significantly lower relative to those on day 1 (mean maximal LH increase (IU/l): baseline, 21.5 ± 10.7; 2 weeks, 10.0 ± 4.3; P < 0.001) (Figure 4d). However, no further significant reductions in LH KP-54 were observed after injection of KP-54 at 4 weeks (mean maximal LH increase: 9.0 ± 4.1 IU/l; P > 0.05 vs. response at 2 weeks), 6 weeks (mean maximal LH increase: 8.9 ± 3.5 IU/l; P > 0.05 vs. response at 2 weeks), and 8 weeks (mean maximal LH increase: 7.9 ± 4.5 IU/l; P > 0.05 vs. response at 2 weeks) (Figure 4d). FSH responses after KP-54 injection showed a pattern similar to those of LH over the 8-week protocol (mean maximal increase in serum FSH after KP-54 injection (IU/l): baseline, 6.4 ± 3.2; 2 weeks, 2.7 ± 0.7, P < 0.001 vs. baseline response; 4 weeks, 2.6 ± 0.7, P > 0.05 vs. response at 2 weeks; 6 weeks, 2.4 ± 0.8, P > 0.05 vs. response at 2 weeks; 8 weeks, 2.7 ± 0.8, P > 0.05 vs. response at 2 weeks) (Figure 4e). Estradiol responses after injection of KP-54 were similar throughout the 8-week protocol of twice-weekly injections (mean maximal increase in serum estradiol after KP-54 injection (pmol/l): baseline, 44.4 ± 19.9; 2 weeks, 39.2 ± 14.8; 4 weeks, 46.2 ± 25.8; 6 weeks, 60.8 ± 17.2; 8 weeks, 20.0 ± 7.5) (Figure 4f).

No significant differences were observed at any stage during the 8-week protocol with respect to the number of follicles, the maximum size of the follicles, the volume of the ovary, and endometrial thickness between subjects who received KP-54 and those who received saline injections (Table 2). More dominant follicles were observed in subjects treated with KP-54 than in subjects treated with saline (six vs. three dominant follicles, respectively); however, no preovulatory follicles were observed in any subject during the study (Table 2). No significant differences were observed in the values of the twice-weekly basal reproductive hormone measurements at any stage during the

Figure 2  Serum reproductive hormone levels in women with HA receiving twice-daily or twice-weekly KP-54 injections for 2 weeks. (a–f) Changes in serum levels of LH, FSH, and estradiol (E2) during 4 h after subcutaneous administration of KP-54 on days 1 and 14 of the of the 2-week dosing regimens (n = 5 per dosing regimen); (a–c) after twice-daily subcutaneous injections of 6.4 nmol/kg of KP-54; (d–f) after twice-weekly subcutaneous injection of 6.4 nmol/kg of KP-54. (g–i) A summary of the effects of subcutaneous injections of KP-54 on days 1 and 14 of each of the different 2-week dosing regimens of KP-54 is shown as the mean AUC for (g) LH, (h) FSH, and (i) estradiol. The data are shown as mean values ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001 vs. day 1. AUC, area under the curve; FSH, follicle-stimulating hormone; HA, hypothalamic amenorrhea; KP-54, kisspeptin-54; LH, luteinizing hormone.
8-week protocol, between subjects receiving saline and those receiving KP-54 injections (Table 3). One subject receiving saline treatment developed a preovulatory LH surge after 8 weeks of saline treatment; however, none of the subjects ovulated during the study.

DISCUSSION

Kisspeptin signaling offers an entirely novel therapeutic target for treating reproductive disorders; however, our understanding of the effects of kisspeptin in patients with infertility is limited. Short-term clinical studies suggest that a single injection of KP-54 safely stimulates the release of reproductive hormones but that its repeated administration causes profound tachyphylaxis in humans. We present novel data suggesting that twice-weekly subcutaneous administration of KP-54 stimulates the release of reproductive hormones but does not restore menstrual cyclicity in women with HA over a 2-month period.

We present detailed data of the time course of desensitization to the effects of KP-54 on reproductive hormone release in women with HA. Our data suggest that desensitization of LH response to twice-daily subcutaneous administration of KP-54 in women with HA occurs gradually over the 14-day injection period. LH responses on day 4 were significantly higher than those on day 14. By contrast, FSH responses dropped to <3 h·IU/l after just 24 h of twice-daily administration of KP-54. It has also been suggested that chronic administration of kisspeptin could be useful as a therapy for sex hormone–responsive cancers because of its inhibitory effects on the release of sex hormones and on tumor metastasis. Our data, which suggest that twice-daily subcutaneous administration of KP-54 leads to downregulation of the hypothalamo–pituitary–gonadal axis, therefore has implications for such kisspeptin-based therapies. Recent evidence suggests that pharmacological blockade of kisspeptin signaling may suppress LH pulsatility rather than basal LH levels.

In view of the tachyphylaxis associated with twice-daily administration of KP-54 (6.4 nmol/kg), we investigated whether lengthening the dose interval to twice-weekly would reduce desensitization. We performed a 2-week pilot study, and the results suggest that gonadotrophin responses are only partially diminished after twice-weekly administration of 6.4 nmol/kg of KP-54. This suggests that subjects with HA remained partially sensitive to KP-54 injection during this twice-weekly regimen. To further assess the efficacy of twice-weekly subcutaneous injection of 6.4 nmol/kg KP-54, we performed a longer, 8-week study. As observed during the pilot study, partial desensitization in the gonadotrophin responses to KP-54 injection was observed during the first 2 weeks of administration. However, these responses did not significantly diminish further beyond this initial 2-week period. Our results therefore suggest that subjects with HA remained partially responsive to KP-54 injection during the 8-week study period.

It is interesting to consider why no significant changes in follicle growth were observed during the 8-week study of twice-weekly KP-54 vs. saline administration. Previous studies of KP-54 administration in humans demonstrated no significant alterations in plasma inhibin in healthy male volunteers. However, in this study, we did observe that FSH responses were lower than LH responses after the administration of kisspeptin. Consistent with this observation, kisspeptin has previously been shown to stimulate the release of LH more potently than it
stimulates the release of FSH when administered to rodents, to healthy male volunteers, or to healthy female volunteers. Furthermore, intravenous bolus injection of gonadotrophin-releasing hormone also stimulates the release of LH release more potently as compared with the release of FSH. We also observed that FSH responses were desensitized more rapidly after KP-54 injection than LH responses were. It is therefore possible that inadequacy in the magnitude and duration of FSH stimulation might have accounted for the lack of detectable ovarian follicular growth observed in this study.

The dose of KP-54 used during this study (6.4 nmol/kg) was selected because it was previously shown to stimulate a mean maximal rise in serum FSH of 9.1 IU, in women with HA. It would be interesting to investigate whether higher doses of twice-weekly KP-54 administration would lead to more potent FSH stimulation and restoration of menstrual cyclicity.

In summary, we present important pharmacologic data regarding the effects of KP-54 on the release of gonadotrophin in a human model of infertility. We have determined the time course of desensitization of LH and FSH responses during twice-daily administration of KP-54 in women with HA. Twice-daily administration of KP-54 is associated with desensitization, a characteristic that may be utilized in the treatment of hormone-sensitive tumors. We have also conducted the first long-term clinical study of KP-54 administration to women with HA. Twice-weekly administration of KP-54 resulted in only partial desensitization, in contrast to complete tolerance achieved with twice-daily administration. However, this protocol of administration of KP-54 did not restore menstrual cyclicity in women with HA. Further work is required to investigate the therapeutic potential of kisspeptin in the treatment of patients with disorders of the reproductive system.

**METHODS**

**Subjects.** Ethical approval for this study was granted by the research ethics committee of Hammersmith and Queen Charlotte’s and Chelsea Hospitals (registration number 05/Q0406/142). Written informed consent was obtained from all the subjects. The study was performed in accordance with the Declaration of Helsinki.

Subjects were recruited through advertisements placed in local newspapers. Women were diagnosed with functional HA if they fulfilled the...
following criteria: body mass index <25 kg/m²; stable body weight for 6 months; age 18–40 years; secondary amenorrhea >6 months; no oral contraceptive pill therapy within the last year; no clinical or biochemical evidence of polycystic ovarian syndrome, thyroid dysfunction, or hyperprolactinemia; a structurally normal hypothalamo–pituitary region (on magnetic resonance imaging) and female reproductive tract (on ultrasound); no therapeutic or recreational drug use; and no systemic disease comorbidity. Twenty subjects with HA were recruited.

### Study 1: determination of the time course of desensitization to the gonadotrophin-inducing effects of KP-54 in women with HA

Five subjects with HA received twice-daily injections of 6.4 nmol/kg KP-54. The dose of KP-54 chosen for this study has previously been shown to result in robust stimulation of gonadotrophin release in healthy female volunteers and in women with HA. On day 1 (the first injection day) and on days 2, 3, 4, and 14 (the last injection day) of the study protocol, blood samples were taken from each subject at specific time points for 4 h after the injection of KP-54. KP-54 was administered subcutaneously at our clinical investigation unit, with the subjects in the supine position. Blood samples were drawn at −30, 0, 15, 30, 45, 60, 75, 90, 120, 150, 180, 210, and 240 min after the injection.

### Study 2: two-week study of release of reproductive hormone in serum in women with HA receiving twice-daily KP-54 injections as compared with those receiving twice-weekly injections

Subjects with HA (n = 5 per group) were randomized to receive, in a single-blinded manner, either twice-daily or twice-weekly injections of 6.4 nmol/kg KP-54 over a 2-week period (see Table 1 for baseline characteristics of the subjects). The subjects attended our clinical investigation unit twice a week, and measurements of reproductive hormone levels were carried out at each of these visits. On day 1 (first day of injection) and day 14 (last day of injection), blood samples were taken from each subject at various time points for 4 h after the injection of KP-54, as described above.

### Study 3: randomized, double-blinded, 8-week study comparing the effects of twice-weekly KP-54 (6.4 nmol/kg) vs. saline in women with HA

A randomized, double-blinded, placebo-controlled, parallel-design study was performed. Ten subjects with HA (see Table 1 for baseline characteristics) were randomized to either saline or 6.4 nmol/kg KP-54 injections (n = 5 per group) twice weekly for 8 weeks. Each subject, in a double-blinded manner. After the first injection of saline or KP-54, blood samples were collected at various time points from each subject for 4 h as described above. This 4-h sampling protocol was repeated at 2, 4, and 6 weeks and on the final day of the study protocol at 8 weeks. Measurements of reproductive hormones were carried out twice a week, and transabdominal ultrasound scans were performed once a week. A summary of the protocol for study 3 is shown in Figure 3. During each study visit, urine was tested in order to exclude pregnancy (Clearview Easy HCG; Inverness Medical Innovations, Waltham, MA). The subjects were interviewed by a physician during each visit to check for the presence of any adverse effects. During the 4-h blood sampling period, diastolic and systolic blood pressure and heart rate were recorded; after the injection (KP-54 or saline), the subjects were repeatedly asked at regular intervals about the presence of any adverse symptoms.

### Table 2 Summarystudy of ultrasound parameters at baseline and during and after 8 weeks of treatment with either saline or kisspeptin in women with hypothalamic amenorrhea

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Study group</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovulation (no. of subjects)</td>
<td>Saline</td>
<td>Kisspeptin-54</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Preovulatory follicle ≥18 mm (no. of subjects)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dominant follicle ≥11 mm (no. of subjects)</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Endometrial thickness (mm)</td>
<td>Weeks 1–2</td>
<td>2.8 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Weeks 3–4</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Weeks 5–6</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Weeks 7–8</td>
<td>3.2 ± 0.7</td>
</tr>
<tr>
<td>Ovarian volume (cm³)</td>
<td>Weeks 1–2</td>
<td>5.7 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>Weeks 3–4</td>
<td>5.2 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>Weeks 5–6</td>
<td>5.4 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>Weeks 7–8</td>
<td>6.1 ± 1.9</td>
</tr>
<tr>
<td>Follicle number per ovary</td>
<td>Weeks 1–2</td>
<td>12 ± 2</td>
</tr>
<tr>
<td></td>
<td>Weeks 3–4</td>
<td>11 ± 3</td>
</tr>
<tr>
<td></td>
<td>Weeks 5–6</td>
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<td></td>
<td>Weeks 7–8</td>
<td>9 ± 4</td>
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<td>Maximum follicular diameter (mm)</td>
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<td>Weeks 3–4</td>
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<td></td>
<td>Weeks 5–6</td>
<td>6.0 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>Weeks 7–8</td>
<td>6.3 ± 0.8</td>
</tr>
</tbody>
</table>

The values given are for subjects randomized to receive twice-weekly injections of saline (n = 5) or of 6.4 nmol/kg KP-54 (n = 5). The change in value of each ultrasound parameter during each 2-week period of the study was based on the results of two separate scans performed during the respective 2-week period: Endometrial thickness, ovarian volume, the number of follicles, and the maximum follicular diameter are shown as mean values ± SEM. KP-54, kisspeptin-54; NS, nonsignificant difference between KP-54 group vs. saline group during each 2-week time period.

### Table 3 Comparison of basal levels of reproductive hormones in serum at baseline and during and after 8 weeks of treatment with either saline or kisspeptin injections

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Study group</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH (IU/l)</td>
<td>Baseline</td>
<td>1.5 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Week 2</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Weeks 3–4</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Weeks 5–8</td>
<td>3.7 ± 2.7</td>
</tr>
<tr>
<td>FSH (IU/l)</td>
<td>Baseline</td>
<td>3.7 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Week 2</td>
<td>2.8 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Weeks 3–4</td>
<td>3.1 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>Weeks 5–8</td>
<td>3.2 ± 0.8</td>
</tr>
<tr>
<td>Estradiol (pmol/l)</td>
<td>Baseline</td>
<td>97 ± 13</td>
</tr>
<tr>
<td></td>
<td>Week 2</td>
<td>91 ± 10</td>
</tr>
<tr>
<td></td>
<td>Weeks 3–4</td>
<td>113 ± 33</td>
</tr>
<tr>
<td></td>
<td>Weeks 5–8</td>
<td>123 ± 37</td>
</tr>
</tbody>
</table>

Mean ± SEM values of the basal hormone levels are given for subject groups randomized to receive twice-weekly injections of saline (n = 5) or of 6.4 nmol/kg KP-54 (n = 5). For each subject, levels of LH, FSH, and estradiol in serum were measured at baseline, and the mean levels were calculated from separate blood tests performed during week 2, weeks 3–4, and weeks 5–8 of the study protocol. FSH, follicle-stimulating hormone; KP-54, kisspeptin-54; LH, luteinizing hormone; NS, nonsignificant difference between KP-54 group vs. saline group during each 2-week time period.
**KP-54 peptide.** KP-54 was synthesized by the Advanced Biotechnology Centre, Imperial College London, and purified using reverse-phase high-performance liquid chromatography. Electrospray mass spectroscopy and amino acid analysis confirmed the identity of the peptide. The peptide was tested for bioactivity and toxicity. The Limulus amoebocyte lysate assay (Associates of Cape Cod, Liverpool, UK) was negative for endotoxin, and the peptide was sterile on culture (Department of Microbiology, Hammersmith Hospital, London, UK). Although kisspeptin-10, -13, -14, and -54 display similar potency in vitro, we used KP-54 because of its higher *in vivo* potency as compared with those of the other kisspeptin fragments.

**Injections.** Vials of freeze-dried saline or KP-54 were reconstituted in 0.5 ml of 0.9% saline. In the studies involving twice-daily injections, the subjects were trained to self-administer these at home. In the studies involving twice-weekly injections, these were administered to the subjects by study investigators during scheduled visits.

**Collection, processing, and analysis of blood samples.** Blood samples for serum analysis were collected in plain serum Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ). Clotted samples were spun for 10 min at 3,000 r.p.m. in a Hettich EBA 20 centrifuge (Hettich International, Tuttlingen, Germany). Sera were separated and stored at −20 °C until analysis. Levels of LH, FSH, estradiol, and progesterone in the sera were measured using automated chemiluminescent immunoassays (Abbott Diagnostics, Maidenhead, UK). Sex hormone–binding globulin was measured using a solid-phase automated enzyme immunoassay (Immolute; Siemens, Llanberis, UK).

**Data analysis.** Data are presented as mean values ± SEM. Hormone profiles during the 4-h blood sample investigations were analyzed using repeated measures two-way analysis of variance with Bonferroni post hoc correction. Pairs of means were analyzed using the unpaired two-tailed *t*-test. Multiple means were compared using one-way analysis of variance with Bonferroni’s multiple-comparison test. In all cases, *P* < 0.05 was considered to be statistically significant.

**ACKNOWLEDGMENTS**

C.N.J. is supported by an NIHR Clinical Lectureship. C.N.J. and G.M.K.N. are supported by Wellcome Trust Research Training Fellowships. W.S. is considered to be statistically significant. In all cases, *P* < 0.05 was considered to be statistically significant.

**CONFLICT OF INTEREST**

The authors declared no conflict of interest.

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The Effects of Kisspeptin-10 on Reproductive Hormone Release Show Sexual Dimorphism in Humans


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Background: Kisspeptin peptides are critical in human reproductive physiology and are potential therapies for infertility. Kisspeptin-10 stimulates gonadotropin release in both male and female rodents. However, few studies have investigated the effects of kisspeptin-10 on gonadotropin release in humans, and none have investigated the effect in women. If kisspeptin is to be useful for treating reproductive disease, its effects in both men and women must be established.

Aim: To compare the effects of kisspeptin-10 administration on reproductive hormone release in healthy men and women.

Methods: Intravenous bolus kisspeptin-10 was administered to men and women (n = 4–5 per group). Subcutaneous bolus and iv infusion of kisspeptin-10 was also administered to female women (n = 4–5 per group). Circulating reproductive hormones were measured.

Results: In healthy men, serum LH and FSH were elevated after iv bolus kisspeptin-10, at doses as low as 0.3 and 1.0 nmol/kg, respectively. In healthy women during the follicular phase of the menstrual cycle, no alterations in serum gonadotropins were observed after iv bolus, sc bolus, or iv infusion of kisspeptin-10 at maximal doses of 10 nmol/kg, 32 nmol/kg, and 720 pmol/kg/min, respectively. In women during the pre-ovulatory phase, serum LH and FSH were elevated after iv bolus kisspeptin-10 (10 nmol/kg).

Conclusion: Kisspeptin-10 stimulates gonadotropin release in men as well as women during the preovulatory phase of menstrual cycle but fails to stimulate gonadotropin release in women during the follicular phase. The sexual dimorphism of the responsiveness of healthy men and women to kisspeptin-10 administration has important clinical implications for the potential of kisspeptin-10 to treat disorders of reproduction. (J Clin Endocrinol Metab 96: E1963–E1972, 2011)
shown to stimulate gonadotropin release in rodents (19–21). In addition, administration of kisspeptin-54 stimulates gonadotropin secretion in humans (22–25). Kisspeptin therefore has the potential to become a novel therapy for treatment of reproductive disorders in humans. The shorter amino acid sequence of kisspeptin-10 makes it simpler and cheaper to synthesize than kisspeptin-54. Future kisspeptin therapies may therefore be based upon kisspeptin-10 rather than kisspeptin-54. It is therefore therapeutically important to determine whether kisspeptin-10 can stimulate reproductive hormone release in healthy men and women. Kisspeptin-10 stimulates gonadotropin release in male rhesus monkeys (13–15). Furthermore, two recent reports have suggested that kisspeptin-10 administration to healthy men stimulates gonadotropin release (26, 27). Although kisspeptin-10 is known to stimulate gonadotropin release in animals (8, 9, 12, 17), there are no published data examining the effects of administering kisspeptin-10 to female primates or humans.

This study aimed to determine the effects of kisspeptin-10 administration on reproductive hormone release in healthy men and, for the first time, in healthy women.

Subjects and Methods

Subjects

The study was conducted with Ethics Committee approval (reference 08/H0707/95) in accordance with The Declaration of Helsinki. Written informed consent was obtained from all subjects. Thirty-five healthy female subjects and 11 healthy male subjects were recruited, using criteria summarized in Supplemental Table 1 (published on The Endocrine Society’s Journals Online web site at http://jcem.endojournals.org).

Study days

Subjects were admitted to our Clinical Investigation Unit and asked to lay supine for the duration of each study. Urine was tested to exclude pregnancy in women (Clearview Easy-HCG; Inverness Medical Innovations Inc., Waltham, MA). All blood samples were analyzed for measurement of serum LH, FSH, estradiol (in women) or testosterone (in men), and plasma kisspeptin immunoreactivity (IR). Heart rate, blood pressure, and the presence of adverse symptoms were recorded at regular intervals.

Study 1: effects of iv bolus injection of saline or kisspeptin-10 in healthy male volunteers

Intravenous bolus injection of 0.9% saline or kisspeptin-10 (at doses of 0.3, 1.0, 3.0, or 10 nmol/kg) was administered at time 0 min. Blood samples were taken at –30, 0, 10, 20, 30, 40, 50, 60, 75, 90, 120, 150, and 180 min (n = 4–5 per group).

Study 2: effects of iv bolus injection of kisspeptin-10 or kisspeptin-54 in healthy female volunteers

Follicular phase of the menstrual cycle

Women between d 2–10 of their menstrual cycle were administered an iv bolus injection of 0.9% saline, kisspeptin-54 (1.0 nmol/kg), or kisspeptin-10 (at doses of 1.0, 3.0, or 10 nmol/kg) at time 0 min, and blood samples were taken at –30, 0, 10, 20, 30, 40, 50, 60, 75, 90, 120, 150, and 180 min (n = 4–5 per group).

Preovulatory phase of the menstrual cycle

Women 15–16 d before their next predicted period received iv bolus injection of 10 nmol/kg kisspeptin-10 as described for the follicular phase (n = 5).

Study 3: effects of sc bolus injection of saline or kisspeptin-10 in healthy female volunteers in the follicular phase of the menstrual cycle

Kisspeptin-10 (at doses 2, 4, 8, 16, or 32 nmol/kg) or 0.9% saline was administered sc at time 0 min, and blood samples were taken at –30, 0, 15, 30, 45, 60, 75, 90, 120, 150, 180, 210, and 240 min (n = 4–5 per group).

Study 4: effects of iv infusion of saline or kisspeptin-10 in healthy female volunteers in the follicular phase of the menstrual cycle

Kisspeptin-10 was dissolved in saline containing gelofusine (5% vol/vol) (B. Braun Medical, Sheffield, UK) and was infused iv over 90 min. During the first 30 min of infusion, the volunteers were administered 20, 50, 90, 180, 360, or 720 pmol/kg/min. The infusion rate for each volunteer was then halved for the remaining 60 min of each infusion. Blood samples were taken at –30, 0, 15, 30, 45, 60, 75, 90, 120, 150, 180, 210, and 240 min.

Study 5: determining the half-life of kisspeptin-10 in healthy female and male volunteers

To determine the plasma half-life of kisspeptin-10 in men, and in women during the follicular and preovulatory phases of the menstrual cycle, frequent blood sampling was performed during iv infusion of 360 pmol/kg/min kisspeptin-10. The protocol was identical to that used in study 4; except that detailed blood sampling was performed at 1-min (from 91–100 min) and 2-min (from 102–120 min) intervals immediately after stopping kisspeptin-10 infusion. Blood samples were assayed for plasma kisspeptin IR. The decay curve of kisspeptin IR was used to calculate the half-time of disappearance (t½) for infused kisspeptin-10 as described previously (22).

Data analysis

Data are presented as mean ± SEM. Time profiles of hormone levels were compared using two-way ANOVA with Bonferroni’s multiple-comparison test. Pairs of means were compared with unpaired t tests (or Mann-Whitney U test if nonparametric), and multiple means of area under curve (AUC) reproductive hormone release were compared using one-way ANOVA with Bonferroni’s multiple-comparison test (or Kruskal-Wallis with Dunn’s multiple-comparison tests if nonparametric). Slopes of linear regression curves were compared using an F test. In all
cases, $P < 0.05$ was considered statistically significant. All data of serum reproductive hormones during treatment are presented as increases in serum levels after injection when compared with preinjection levels.

**Results**

Baseline characteristics of the subjects recruited to the study are summarized in Table 1.

**Study 1: effects of iv bolus injection of saline or kisspeptin-10 in healthy male volunteers**

Plasma kisspeptin IR was elevated after iv bolus injection of kisspeptin-10 at all doses in healthy male volunteers (Fig. 1, A and E). The highest plasma kisspeptin IR was observed after 10 nmol/kg kisspeptin-10 (mean AUC kisspeptin IR was 700 ± 160 h·pmol/liter, $P < 0.001$ vs. saline). At this dose, mean peak kisspeptin IR (3350 ± 72.5 pmol/liter) was observed 10 min after injection, and plasma kisspeptin IR returned to undetectable levels 50 min after injection. Serum LH was elevated significantly after administration of each tested dose of iv bolus kisspeptin-10 injection compared with saline (Fig. 1, B and F). Peak stimulation of serum LH was observed 30–40 min after injection, and levels of serum LH gradually returned to baseline 180 min after injection (Fig. 1B). Maximal stimulation of LH was observed after iv bolus 10 nmol/kg kisspeptin-10 (mean AUC LH increase was 6.1 ± 1.3 IU/liter, $P < 0.001$ vs. saline) (Fig. 1F). Serum FSH was significantly increased compared with saline injection after iv bolus injection of 1.0 or 3.0 nmol/kg kisspeptin-10 (Fig. 1, C and G). Serum testosterone was significantly increased compared with saline injection after iv bolus injection of 0.3 or 1.0 nmol/kg kisspeptin-10 (Fig. 1, D and H). Serum levels of testosterone at these doses steadily increased to peak levels 150–180 min after injection (Fig. 1D).

**Study 2: effects of iv bolus injection of saline or kisspeptin-10 or kisspeptin-54 in healthy female volunteers**

**Follicular phase of the menstrual cycle**

Plasma kisspeptin IR was elevated after iv bolus injection of kisspeptin-10 at all doses in healthy female volunteers during the follicular phase of menstrual cycle (Fig. 2, A and E). The highest plasma kisspeptin IR was observed after iv bolus injection of 10 nmol/kg kisspeptin-10 (mean AUC kisspeptin IR in women during follicular phase was 527 ± 108 h·pmol/liter, $P < 0.01$ vs. saline); this was lower when compared with kisspeptin IR after injection of the same dose of kisspeptin-10 to men, but this difference was not significant ($P = 0.42$ vs. men). At this dose, mean peak kisspeptin IR (2638 ± 302 pmol/liter) was observed 10 min after injection, and plasma kisspeptin IR returned to undetectable levels 50 min after injection. Gonadotropin release was elevated significantly after iv bolus injection of 1 nmol/kg kisspeptin-54 [mean AUC increase (in h·IU/liter): 27.0 ± 11.8 (LH), $P < 0.05$ vs. saline, and 7.9 ± 4.1 (FSH), $P < 0.05$ vs. saline]. Unexpectedly, no significant changes in serum levels of reproductive hormones were observed after iv bolus injection of kisspeptin-10 at doses up to 10 nmol/kg (Fig. 2, B–D and F–H).

**Preovulatory phase of the menstrual cycle**

Kisspeptin IR was elevated significantly in women during the preovulatory phase after kisspeptin-10 injection compared with saline, and this elevation was nonsignificantly different when compared with kisspeptin IR after the same dose of kisspeptin-10 in follicular-phase women or men (mean AUC kisspeptin IR in preovulatory phase was 320 ± 56 h·pmol/liter, $P = 0.13$ vs. follicular phase, and $P = 0.06$ vs. men) (Fig. 2E). Serum LH and FSH were elevated significantly after iv bolus injection of 10 nmol/kg kisspeptin-10 in women during the preovulatory phase of the menstrual cycle [mean AUC increase was 30.3 ± 7.7 h·IU/liter (LH), $P < 0.05$ vs. saline, and 6.9 ± 0.9 h·IU/liter (FSH), $P < 0.01$ vs. saline] (Fig. 2, B, C, F, and G); however, serum estradiol was not altered significantly (mean AUC estradiol increase was 111 ± 96 h·pmol/liter, $P = 0.14$ vs. saline) (Fig. 2, D and H).

<table>
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<tr>
<th>TABLE 1. Baseline characteristics of healthy male and female volunteers administered kisspeptin-10</th>
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<td><strong>Baseline characteristic</strong></td>
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<td>Age (yr)</td>
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<td>Length of menstrual cycle (d)</td>
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<td>LH (IU/liter)</td>
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Female endocrine profiles are presented during the follicular and preovulatory phases of the menstrual cycle. Data are shown as mean ± SEM.

a $P < 0.05$ vs. follicular phase of the menstrual cycle.

b $P < 0.01$ vs. follicular phase of the menstrual cycle.
Study 3: effects of sc bolus injection of saline or kisspeptin-10 in healthy female volunteers in the follicular phase of the menstrual cycle

Plasma kisspeptin IR was significantly elevated during the 4 h after sc injection of kisspeptin-10 at doses of 4 nmol/kg and higher, when compared with saline (Fig. 3A). The highest plasma kisspeptin IR after sc injection was observed after injection of 32 nmol/kg kisspeptin-10 (mean AUC kisspeptin IR was 201 \pm 16 \text{ pmol/liter}, P < 0.001 vs. saline).

No significant changes in serum reproductive hormone levels were observed after sc bolus injection of kisspeptin-10 at any dose (Fig. 3, B–H).

Study 4: effects of iv infusion of kisspeptin-10 in healthy female volunteers in the follicular phase of the menstrual cycle

Intravenous infusion of peptides delivers the peptide directly into the circulation, avoiding possible degradation in sc tissue, and results in a sustained increase in circulating levels of the administered peptide.

Plasma kisspeptin IR increased during iv infusion of kisspeptin-10 at all doses (Fig. 4A). The highest plasma kisspeptin IR was observed during iv infusion of 720 pmol/kg/min kisspeptin-10 (mean AUC kisspeptin IR was 2518 \pm 100 \text{ h pmol/liter}). All iv infusion doses of kisspeptin-10 were associated with a higher mean plasma kisspeptin IR than the highest studied sc dose of kisspeptin-10 (32 nmol/kg, mean AUC kisspeptin IR was 201 \pm 16 \text{ h pmol/liter}). No significant changes in serum reproductive hormone levels were observed after iv infusion of any dose of kisspeptin-10 in healthy female volunteers in the follicular phase of the menstrual cycle (Fig. 4, B–H).

Study 5: pharmacokinetic profile of kisspeptin IR during iv infusion of kisspeptin-10 in healthy male and female volunteers

To determine the plasma half-life of kisspeptin-10, frequent blood sampling was performed in women during the follicular and preovulatory phases of menstrual cycle, and in men, after cessation of an iv infusion of 360 pmol/kg/min kisspeptin-10 (Fig. 5). When plotted on a natural log scale, linear regression slopes were not significantly different among the three groups (F = 0.099; degrees of Freedom numerator = 2; degrees of Freedom denominator = 111; P = 0.91), and plasma half-lives of kisspeptin-10 were calculated as 3.8 \pm 0.3 \text{ min (men)}, 4.1 \pm 0.4 \text{ min (follicular-phase women)}, and 4.1 \pm 0.4 \text{ min (preovulatory-phase women)} (Fig. 5E).

Discussion

These studies reveal a previously unknown sexual dimorphism in responsiveness to kisspeptin-10 administration...
in healthy men and women. Numerous studies in animals have demonstrated that kisspeptin-10 robustly stimulates gonadotropin release, thus implicating kisspeptin signaling as a potential therapeutic target for treating female patients with infertility (8, 9, 12, 17). Kisspeptin-10 stimulates gonadotropin release in male rhesus monkeys (13–15), and recent reports have suggested that kisspeptin-10 administration stimulates gonadotropin release in healthy men (26, 27). However, the effects of administration of kisspeptin-10 in female primates or women have not previously been studied.

We observed that iv bolus injection of kisspeptin-10 robustly stimulated LH release in healthy men at all doses tested. These results are consistent with our previous observation that administration of the longer form of kisspeptin, kisspeptin-54, stimulates reproductive hormone release in men (22). Furthermore, two recent reports have shown that iv injection of kisspeptin-10 potently stimulates LH release in healthy male volunteers at doses similar to those used in this study (26, 27) and that prolonged infusion of kisspeptin-10 significantly stimulates LH pulsatility in healthy male volunteers (27).

Studies in rodents suggest that kisspeptin-10 stimulates gonadotropin release in both males and females (8, 9). Furthermore, we observed that the kisspeptin-10 peptide used in this study stimulated LH release in female adult mice (Supplemental Fig. 1), although with a much lower potency when compared with kisspeptin-54. We were therefore surprised to observe that doses of kisspeptin-10 identical to those used in men failed to stimulate reproductive hormone release in healthy female volunteers during the follicular phase of the menstrual cycle when administered as an iv bolus injection. It is possible that the lack of effect of iv administration of kisspeptin-10 to stimulate reproductive hormone release in women in the follicular phase of their menstrual cycle was due to the short period of time that circulating kisspeptin-10 levels were elevated after iv bolus administration. Subcutaneous bolus administration of kisspeptin-10 is likely to result in a longer period of elevated circulating levels of kisspeptin-10 than iv bolus administration (23). Furthermore, we have previously determined that sc bolus injection of kisspeptin-54 at doses as low as 0.4 nmol/kg potently stimulates serum LH in healthy women during the follicular phase of the menstrual cycle (23). However, serum gonadotropin levels were not elevated by sc bolus injection of kisspeptin-10 in healthy women in the follicular phase of the menstrual cycle, despite elevations in plasma kisspeptin IR for up to 90 min after injection.

FIG. 2. Plasma kisspeptin IR and serum reproductive hormone levels after iv bolus injection of kisspeptin-10 to healthy female volunteers. A–D, Time profiles for plasma kisspeptin IR (A) and changes in serum LH (B), FSH (C), and estradiol (D) during 4 h after iv bolus injection of saline, kisspeptin-10 (KP10), or kisspeptin-54 (KP54) to healthy female volunteers. For 10 nmol/kg KP10 vs. saline: *, P < 0.05; **, P < 0.01; ***, P < 0.001. For 3 nmol/kg KP10 vs. saline: **, P < 0.01. For 10 nmol/kg KP54 vs. saline: **, P < 0.01; ***, P < 0.001. E–H, AUC for plasma kisspeptin IR (E) and changes in serum LH (F), FSH (G), and estradiol (H) during 4 h after iv bolus injection of saline or kisspeptin-10 to healthy female volunteers. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Data are shown as mean ± sem. E2, Estradiol; Preov, preovulatory phase of the menstrual cycle.
have been due to breakdown of kisspeptin-10 in the sc tissue. Intravenous infusion of peptides delivers the peptide directly into the circulation, avoiding possible degradation in sc tissue, and results in a sustained increase in circulating levels of the administered peptide. However, serum gonadotropin levels were not elevated during iv infusion of kisspeptin-10 to healthy female volunteers in the follicular phase of the menstrual cycle, despite markedly raised plasma levels of kisspeptin IR up to concentrations of 2000 pmol/liter. Our data therefore suggest that women in the follicular phase of the menstrual cycle are markedly less responsive to kisspeptin-10 administration than men. The underlying mechanism for this observation is unclear. However, sexual dimorphism of hypothalamic kisspeptin signaling pathways has been demonstrated in rodents (28–30).

It is important to consider whether the contrasting effects of kisspeptin-10 in men and women may have been attributable to factors other than differential sensitivity to the peptide. We observed that levels of plasma kisspeptin IR appeared slightly lower in women when compared with men when identical weight-adjusted doses of kisspeptin-10 were administered. It is possible that levels of kisspeptin-10 may have been modified by factors known to differ between the sexes, such as body fat content or clearance of peptide from the circulation (19). However, it is noteworthy that only a marginal elevation of plasma kisspeptin IR (approximately 10 h⁻¹ pmol/liter) was necessary to stimulate significant LH secretion in men after injection of kisspeptin-10 (0.3 nmol/kg iv bolus); by contrast, a 200-fold greater elevation in plasma kisspeptin IR failed to stimulate LH release in women during the follicular phase of the menstrual cycle. Furthermore, we observed no significant differences in the plasma half-lives of kisspeptin-10 between men and women. It is therefore unlikely that the striking contrast between male and female responsiveness to kisspeptin-10 administration reflects differences in metabolism of kisspeptin-10. We observed nonsignificant reductions in serum LH (P = 0.19) and FSH (P = 0.91) after saline injection in men when compared with saline injection in women. Although both men and women underwent identical study protocols, it is possible that minor differences in factors inhibiting reproductive function such as stress may be greater in the male group. However,
such a difference would not explain why men are more responsive to kisspeptin-10 than women in the follicular phase of the menstrual cycle. In the current study, we observed that exogenous kisspeptin-54 stimulated significant gonadotropin secretion in women during the follicular phase of the menstrual cycle, despite a failure of exogenous kisspeptin-10 to stimulate gonadotropin secretion at a 10-fold higher molar dose. Furthermore, kisspeptin-54 injection stimulated LH in female mice more potently when compared with kisspeptin-10, and it has been previously observed that kisspeptin-52 (31) and kisspeptin-54 (21) stimulate LH more potently when compared with kisspeptin-10 in male rats. Collectively, these data suggest that the greater potency of kisspeptin-54 when compared with kisspeptin-10 may explain why exogenous kisspeptin-54 injection (but not exogenous kisspeptin-10) stimulates reproductive hormone release in women during the follicular phase of the menstrual cycle. The differences between kisspeptin-10 and kisspeptin-54 may be a consequence of the rapid breakdown of kisspeptin-10 in the circulation. The in vivo plasma half-life of iv kisspeptin-10 in this study was calculated to approximately 4 min, which is 7-fold shorter than the calculated in vivo plasma half-life of kisspeptin-54 (22).

It is interesting to consider whether men and women have differential responses to kisspeptin-54 (as they appear to be to kisspeptin-10) based on previous studies. Intravenous infusion of kisspeptin-54 at doses as low as 1.2 nmol (0.25 pmol/kg/min) stimulate LH secretion in healthy men (22). By comparison, sc bolus injection of kisspeptin-54 at doses of 28 nmol (0.4 nmol/kg) or more stimulate LH secretion in healthy women during the follicular phase of the menstrual cycle (23); furthermore, this study suggests that iv bolus injection of approximately 60 nmol (1 nmol/kg) kisspeptin-54 stimulates significant LH secretion in these women. These data demonstrate that, unlike kisspeptin-10, kisspeptin-54 can stimulate reproductive hormone release in women in the follicular phase of their menstrual cycle.

In the current study, gonadotropin responses during kisspeptin-10 infusion appeared more variable than after iv or sc bolus injections. These alterations in gonadotropin secretion did not appear to be related to either the dose or to the onset of kisspeptin infusion; however, it is possible that subtle effects of kisspeptin-10 infusion on gonadotropin release were not detected in the current study protocol.

We have previously demonstrated that women in the preovulatory phase of the menstrual cycle are significantly more sensitive to the effects of kisspeptin-54 on gonadotropin release when compared with women in the follicular phase of the menstrual cycle (23). In keeping with these observations, iv bolus injection of kisspeptin-10 significantly stimulated LH and FSH release in women dur-
ing the preovulatory phase of the menstrual cycle in the current study. Because we observed that kisspeptin-10 has a similar pharmacokinetic profile in both follicular and preovulatory phases of the menstrual cycle, our results suggest that women have heightened sensitivity to kisspeptin-10 during the preovulatory phase of the menstrual cycle. In female rodents, c-fos expression within kisspeptin neurons and levels of kiss1 expression are increased within the anteroventral periventricular nucleus of the hypothalamus immediately before ovulation (35). Furthermore, levels of kiss1r expression are increased in rat hypothalamic fragments at diestrus when compared with proestrus (36). It is therefore possible that women in the preovulatory phase of the menstrual cycle have heightened responsiveness to kisspeptin-10 administration when compared with women during the follicular phase of the menstrual cycle.

In the current study, we observed that kisspeptin-10 stimulated LH secretion more potently than FSH. This is consistent with our previous studies of kisspeptin-54 administration in healthy men and women. After iv bolus kisspeptin-10 injection, peak FSH secretion was observed 45–150 min after injection, which was later than peak LH secretion (30 min after injection); this phenomenon was also observed after sc bolus injection of kisspeptin-54 in healthy women (23). Our results therefore suggest that both kisspeptin-10 and -54 stimulate LH secretion more potently and more rapidly than FSH.

It is interesting to consider why we did not observe any consistent stimulation of testosterone secretion after iv bolus kisspeptin-10 injection in healthy men when compared with the robust increases in serum LH and FSH observed at all tested doses. Significant increases in serum testosterone were observed only at 0.3 and 1.0 nmol/kg iv bolus kisspeptin-10, but these rises were marginal (no more than 10 hnmol/liter above baseline). Furthermore, iv bolus injection of kisspeptin-10 stimulated gonadotropin release in women during the follicular phase of the menstrual cycle but did not increase serum estradiol during 3 h after injection. Our previous data suggest that at least 4 h are required for serum levels of sex steroids to peak after a sc bolus injection of kisspeptin-54 (23–25). A longer period of blood sampling after injection may have revealed more pronounced alterations in sex steroid secretion in subjects after injection of kisspeptin-10. We observed that serum gonadotropins almost returned to baseline levels within 3 h after iv bolus injection of kisspeptin-10; it is possible that iv bolus injection of kisspeptin-10 has a duration of action inadequate to stimulate significant gonadal sex steroid release.

In the current study, all tested iv bolus doses of kisspeptin-10 (0.3–10 nmol/kg) were associated with similar degrees of gonadotropin secretion in healthy male subjects. A recent study by George et al. (27) suggests that kisspeptin-10 stimulates serum LH secretion at doses as low as 0.01 µg/kg (equivalent to 0.008 nmol/kg), with a maximal response seen at 1 µg/kg (0.8 nmol/kg) in men. Taking these data into consideration, all doses of kisspeptin-10 selected during our study may have stimulated near-maximal levels of gonadotropin secretion in healthy male volunteers. Interestingly, George et al. (27) observed that the increase in serum LH at 3 µg/kg (2.4 nmol/kg) was lower than the increase in serum LH at 1 µg/kg, which they speculated was attributable to tachyphylaxis, a phenomenon that we have previously observed after chronic kiss-
peptin-54 injections (24, 25, 27). We therefore cannot exclude that the similarity between LH responses at doses between 0.3 and 10 nmol/kg kisspeptin-10 in men might be explained in part by tachyphylaxis to kisspeptin-10 at the higher tested doses. Additional studies are required to investigate these observations.

In summary, this is the first clinical study to report the effects of kisspeptin-10 administration on gonadotropin release in women and to compare the effects of kisspeptin-10 between men and women. Kisspeptin-10 robustly stimulates gonadotropin release in men but fails to stimulate gonadotropin release in healthy female volunteers in the follicular phase of the menstrual cycle when administered by iv bolus injection, sc bolus injection, or iv infusion. These experiments reveal sexual dimorphism in the responsiveness of healthy human volunteers to kisspeptin-10 administration. These findings have important clinical implications for the potential therapeutic use of kisspeptin-10 to treat disorders of reproduction.

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