THE UNIVERSITY *of* EDINBURGH

Functional characterisation and translational applications of kisspeptin-10.

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To Kavitha, Lizzie and Liam

Who did miss many a kiss

Whilst away I was, in the world of *KiSS*

Preface

This thesis arises from translational clinical studies carried out at the MRC Human Reproductive Sciences Unit supported by an MRC Experimental Medicine grant to Professor Robert Millar with Professor Richard Anderson as co-applicant between January 2009 and April 2011 when the Unit was closed. The remainder of the grant over the next 9 months was utilised in the MRC/University of Edinburgh Centre for Reproductive Health.

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Declaration

I declare

- That the thesis has been composed by myself (and edited in parts by my supervisors)
- That the work is my own
- And that this work has not been submitted for any other degree or professional qualification.

Jyothis Thomas George

List of publications related to this thesis

1. George, J. T., Millar R.P, Anderson R.A (2010). "Hypothesis: kisspeptin mediates male hypogonadism in obesity and type 2 diabetes." Neuroendocrinology 91(4): 302-307.
2. George, J. T., Veldhuis JD, Roseweir AK, Newton CL, Faccenda E, Millar RP, Anderson R.A. (2011). "Kisspeptin-10 Is a Potent Stimulator of LH and Increases Pulse Frequency in Men." Journal of Clinical Endocrinology & Metabolism 96(8): E1228-E1236.
3. Young J, George JT, Tello JA, Francou B, Bouligand J,Guiochon-Mantel A, Brailly-Tabard S, Anderson R.A, Millar R.P. Kisspeptin restores pulsatile Gonadotropin Secretion in Patients with Neurokinin B Signalling Deficiencies: Therapeutic Implications. Neuroendocrinology (*In Press*).
4. George JT, Veldhuis JD, Tena-Sempre M, Millar RP, Anderson RA. Kisspeptin-10 increases LH pulse frequency and normalises serum testosterone in hypogonadotropic hypogonadal men with type 2 diabetes. (*Manuscript in review*).
5. George, J. T., Anderson RA, Millar R.P. LH Reponses to Kisspeptin-10 in Women Exposed to Different Sex Steroid Environments. (*Manuscript in review*).

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* Indicates colour figures

Abbreviations

ANOVA	Analysis of Variation
AR	Androgen Receptor
ARC	Arcuate Nucleus
AUC	Area Under curve
AVPV	Anteroventral Periventricular Nucleus
CSF	Cerebrospinal Fluid
CV	Co-efficient of Variability
DHT	Dihydrotestosterone
ELISA	Enzyme-linked Immunosorbent Assay
ERα	Estrogen Receptor Alpha
ERβ	Estrogen Receptor Beta
FAS	Free Alpha Subunit
FSH	Follicle Stimulating Hormone
GH	Growth Hormone
GMP	Good Manufacturing Practice
GnIH	Gonadotropin Inhibitory Hormone
GnRH	Gonadotropin Releasing Hormone
GPR	G-Protein Coupled Receptor
HCG	Human Chorionic Gonadotropin
HPLC	High Performance Liquid Chromatography
ICV	Intracerebroventricilar
IHH	Idiopathic Hypogonadotropic Hypogonadism
IV	Intravenous

KNDy	Kisspeptin Neurokinin B Dynorphin Neurons
Kp	Kisspeptin
Kp-IR	Kisspeptin Immunoreactivity
LH	Luteinising Hormone
MBH	Median Basal Hypothalami
MRC	Medical Research Council
NKB	Neurokinin B
NKR3	Neurokinin Receptor 3
PeN	Periventricular Nucleus
PoA	Pre-optic Area
PR	Progesterone Receptor
RIA	Radioimmuno Assay
RP3V	Rostral Periventricular Region of Third Ventricle
SCN	Suprachiasmatic Nucleus
SD	Standard Deviation
SEM	Standard Error of Mean
T	Testosterone
T2DM	Type 2 Diabetes Mellitus
TSH	Thyroid Stimulating Hormone
UoE	University of Edinburgh

Abstract

Background: Kisspeptins, recently discovered hypothalamic neuropeptides encoded by the *KISS1* gene, are essential for normal pubertal development and are modulated by diverse endocrine, metabolic and environmental signals. Exogenous kisspeptin administration potently stimulates LH secretion - by direct action on GnRH neurons while kisspeptin antagonists inhibit pulsatile LH secretion. Human studies of kisspeptin had hitherto used kisspeptin-54 that is cleaved further and the smallest bioactive form is a decapeptide (kisspeptin-10) with a shorter half-life. Kisspeptin-10 is thus putatively more attractive in studies assessing LH pulsatility and is also the basis for the development of antagonists.

Unmet clinical needs: Decreased LH pulse frequency is the central pathology in pubertal delay, late-onset male hypogonadism and hypothalamic amenorrhoea. Manipulation of LH pulse frequency also has therapeutic potential in contraception, PCOS and sex-steroid dependant diseases such as endometriosis and prostatic hyperplasia.

Hypothesis: That exogenous kisspeptin-10 enhances pulsatile LH secretion in healthy men and in patients with reproductive disorders associated with decreased pulse frequency.

Research strategy: A first-in-human dose escalation study of kisspeptin-10 was performed in men and subsequently replicated in women. An intravenous infusion regime was optimised in healthy men and subsequently applied to hypogonadal patients. Specific questions were addressed sequentially as summarised below with key results.

Dose escalation study:

Question: Does kisspeptin-10 stimulate LH secretion in men?

Findings: Six iv bolus doses (0.01 to 3 μg/kg) of GMP kisspeptin-10 and vehicle were administered at least a week apart to six healthy men. Rapid increase in LH, with peak concentrations was seen by 45 min post injection in all volunteers. There was a clear dose-dependent increase in LH concentrations in response to kisspeptin-10 ($P < 0.0001$). Area-Under-Curve analysis over 60 min following kisspeptin-10

administration showed 0.3 and 1μg/kg doses to be maximally stimulatory (P <0.01) with a reduced response at 3 μg/kg.

Assessing the effect of steroid milieu:

Question: Steroid feedback is central to the regulation of LH secretion: what effect does the steroid milieu have on LH responses to kisspeptin-10?

Findings: The response to iv kisspeptin-10 (0.3μg/kg,) in the normal follicular phase (n=10) was compared with that in the presence of low endogenous sex steroids/high LH secretion (6 postmenopausal women) and in women taking combined contraceptive therapy (n=8) with suppressed LH secretion. Despite widely varying baseline secretion, LH increased significantly following kisspeptin-10 administration in the follicular phase (6.3±1.2 to 9.4±1.3 IU/L P=0.006), postmenopausal (35.3±2.8 to 44.7±3.4 IU/L P=0.005), etonogestrel (4.6±0.2 to 7.5±0.9 IU/L, P=0.02), and COCP groups (2.2±0.9 to 3.7±1.4 IU/L P<0.001).

Pulse frequency study:

Question: GnRH and LH secretion are pulsatile: can kisspeptin-10 enhance LH pulsatility?

Findings: Four healthy men attended our clinical research facility for two visits five days apart for 10-min blood sampling. At the first visit, baseline LH pulsatility was assessed over a 9-hour period. During the second visit, an infusion of kisspeptin-10 was administered for 9 hours at 1.5μg/kg/hr after an hour of baseline sampling. LH pulse frequency increased in all subjects, with a mean increase from 0.7±0.1 to 1.0±0.2 pulses/hr (P = 0.01), with resultant increase in mean LH from 5.2±0.8 IU/L at baseline to 14.1±1.7 IU/L (P <0.01).

High dose, longer duration infusion study:

Question: Can kisspeptin-10 enhance testosterone secretion?

Findings: Four healthy men attended our clinical research facility for a 34-hour supervised stay. Blood samples were collected at 10 min intervals for two 12 hour periods on consecutive days and hourly overnight. After 10.5 hours of baseline sampling a continuous intravenous infusion of kisspeptin-10 (4μg/kg/hr) was

maintained for 22.5 hrs. Mean LH increased from 5.5±0.8 at baseline to 20.9±4.9 IU/L (P <0.05) and serum testosterone increased from 16.6±2.4 to 24.0±2.5 nmol/L (P <0.001).

Translational studies in hypogonadal men with type 2 diabetes

Question: Can kisspeptin-10 normalise testosterone secretion in hypogonadal men?

Findings: Five hypogonadal men with T2DM (age 33.6±3 yrs, BMI 40.6±6.3, testosterone 8.5±1.0 nmol/L, LH 4.7±0.7 IU/L, HbA1c <8 %, duration of diabetes <5 yrs) and seven age matched healthy men were studied. Kisspeptin-10 was administered intravenous (0.3 μg/kg) with frequent (10-min) blood sampling. Mean LH increased in controls (5.5±0.8 to 13.9±1.7 IU/L P <0.001) and in T2DM (4.7±0.7 to 10.7±1.2 IU/L P=0.02) with comparable ΔLH (P=0.18).

Baseline serum sampling for LH at 10-min intervals and hourly testosterone measurements were performed subsequently in four T2DM men for 12 hours. An intravenous infusion of kisspeptin-10 (4 μg/kg/hr) was administered 5 days later for 11 hours, with increases in serum LH (3.9±0.1 IU/L to 20.7±1.1 IU/L (P=0.03,) and testosterone (8.5±1.0 to 11.4±0.9 nmol/L, P=0.002). LH pulse frequency at baseline was lower in hypogonadal men with diabetes (0.6±0.1 vs. 0.8±0.1 pulses/hr, P=0.03) and increased to 0.9±0 pulses/hr (P=0.05).

Translational studies in pubertal delay:

Question: Defective Neurokinin B activity is associated with pubertal delay and the hierarchical interactions between kisspeptins and Neurokinin B remain to be elucidated: can kisspeptin-10 stimulate LH secretion with impaired Neurokinin B signalling?

Findings: Four patients with *TAC3* or *TACR3* inactivating mutations presenting with delayed puberty were admitted for two 12 hr blocks of blood sampling every 10 min with vehicle (saline) or kisspeptin-10 (1.5 μg/kg/hour) infused intravenously. Mean LH and LH pulses frequency increased with kisspeptin-10 (P<0.05). However, four patients with Kallmann syndrome (with defective GnRH neuron migration), studied in parallel, did not respond, suggesting a potential diagnostic application for kisspeptin-10 in pubertal dysfunction.

Conclusions

In first-in-man studies of kisspeptin-10, it was demonstrated that endogenous LH pulse frequency can be enhanced in healthy men. The therapeutic potential of this finding in common reproductive endocrine disorders associated with decreased LH pulse frequency, i.e., late-onset male hypogonadism and pubertal dysfunction, was suggested in subsequent studies. Furthermore, kisspeptin signalling occurs upstream of GnRH neurons and is independent of Neurokinin B signalling in the central regulation of the hypothalamic-pituitary-gonadal axis.

1 Introduction

1.1 GnRH – the principal regulator of reproduction

1.1.1 The discovery of GnRH and evolutionary aspects

Gonadotropin Releasing Hormone (GnRH) was first isolated and structurally identified from porcine hypothalami four decades ago as a decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly·NH2) (Baba, Matsuo et al. 1971; Matsuo, Arimura et al. 1971; Schally, Arimura et al. 1971; Schally, Arimura et al. 1971). This decapeptide was shown to potently stimulate LH and FSH release from the pituitary in a number of mammalian species (Schally, Arimura et al. 1971). Hypophysiotropic properties of the naturally occurring peptide was also demonstrated to be comparable with those observed with its synthetic analogues (Schally, Arimura et al. 1971). Early work referred to this peptide as Luteinising Hormone Releasing Hormone (LH-RH), but has since been widely referred to as GnRH to reflect the dual stimulatory role – although many authors still refer to it as LH-RH (Schally 2000). The pivotal discovery of GnRH was acknowledged with the Nobel Prize for Medicine in 1977 (Nobelprize.org 2009).

Diverse forms of GnRH and its receptor exist among vertebrates, with over twenty primary structures relatively well conserved across species. This suggests that the GnRH signalling system developed early in the evolutionary sequence (Millar 2005; Okubo and Nagahama 2008). As the mammalian decapeptide was the first primary GnRH structure to be identified, it is referred to by convention as GnRH I (Miyamoto, Hasegawa et al. 1984). Another vertebrate GnRH sequence was first identified from chicken brain (pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH2) structurally distinct from GnRH I - now referred to as GnRH II (Miyamoto, Hasegawa et al. 1984; Millar 2005). Subsequently, a third form has also been described in fish and is now referred to as GnRH III (White, Bond et al. 1994; Millar 2005). In mammals, hypophysiotropic functions are limited to GnRH I (Gault, Maudsley et al. 2003) and hence in the human context GnRH I continues to be referred to as GnRH – hence all references to GnRH in this thesis refer to GnRH I.

1.1.2 Neuroanatomy of GnRH neurons and clinical significance

GnRH neurons originate in the medial olfactory placode during embryological development and migrate along the olfactory bulb and tract to their final positions within the hypothalamus (Balasubramanian, Dwyer et al. 2010; Mitchell, Dwyer et al. 2011). Defective GnRH migration leads to the clinical syndrome of Kallmann Syndrome – characterised by GnRH deficiency and anosmia (Kallmann, Schönfeld et al. 1943-1944; Mitchell, Dwyer et al. 2011). A number of factors contributing to this migratory process have been identified. Anosmin-1 (the product of *KAL* gene) (Cariboni, Pimpinelli et al. 2004), neuropilins (Cariboni, Hickok et al. 2007) and Leukaemia Inhibitory factor (Magni, Dozio et al. 2007) are some of the various chemotactic factors described, along with mutations in *FGFR* (Dode, Levilliers et al. 2003). Mutations in *PROK1 and PROK2* lead to hypogonadotropic hypogonadism without anosmia, suggesting that factors other than suboptimal migration can also lead to functional deficiencies in human GnRH function (Pitteloud, Zhang et al. 2007; Martin, Balasubramanian et al. 2011; Mitchell, Dwyer et al. 2011).

GnRH cell bodies are located in the medial preoptic area and the arcuate nucleus of the hypothalamus (ARC) and form a neural network with projections to the median eminence (Clifton and Steiner 2009). GnRH is secreted from the median eminence into the fenestrated capillaries of portal circulation and therein carried to the anterior pituitary (Clifton and Steiner 2009). Studies in male and female rats have quantified the number of GnRH neurons in the forebrain to be around 1300 (Wray and Hoffman 1986). This number has been extrapolated to the human setting with estimates ranging between 1000 neurons (Millar 2005) and 1500 neurons (Balasubramanian, Dwyer et al. 2010).

The co-location of GnRH neurons in the ARC with central neuronal regulators of other key physiological functions allows the GnRH neuronal network to be regulated by a range of neuroendocrine and metabolic inputs. Moreover, GnRH neurons receive afferent signals from the suprachiasmatic nucleus (SCN) – an area of the hypothalamus that expresses *clock* genes with circadian rhythmicity as well as Estrogen Receptor Alpha (ERα) (Clifton and Steiner 2009).

1.1.3 GnRH Pulsatility and interspecies differences

Two distinct modes of GnRH secretion have been described – the pulsatile mode and the surge mode (Maeda, Ohkura et al. 2010). Pulsatile mode refers to the episodic release of GnRH observed in males and in females during most of the menstrual cycle, where there are distinct pulses of GnRH secretion into the portal circulation with undetectable GnRH concentrations in portal serum during inter-pulse intervals. During the preovulatory surge in LH secretion, there appears to be persistent presence of GnRH in the portal circulation – this pattern of GnRH secretion is referred to as the 'surge mode' of GnRH secretion (Maeda, Ohkura et al. 2010). However, it is unclear whether this persistence of GnRH in portal serum in between pulses is representative of continuous GnRH secretion or whether it is merely an artefact induced due to insufficiently frequent sampling and altered dilution/elimination kinetics in the context of a markedly increased pulse frequency (Moenter, Anthony DeFazio et al. 2003).

1.1.3.1 Pulsatile mode of LH secretion

The episodic nature of LH secretion was first suspected when Knobil and co-workers at the University of Pittsburgh, attempting to validate a radioimmunoassay to measure serum LH in the Rhesus monkey, noticed marked variations in hormone concentrations measured (Dierschke, Bhattacharya et al. 1970; Knobil 1981). Experimental studies with frequent blood sampling regime were set up to investigate this phenomenon (Dierschke, Bhattacharya et al. 1970). In adult female Rhesus monkeys ovariectomised 0.5-7 months prior to experimentation, frequent (10-30 min) intra-cardiac sampling demonstrated that circulating LH concentrations showed pulsatile rise, with individual pulses occurring around an hour apart (Dierschke, Bhattacharya et al. 1970). When individual monkeys were studied repetitively, their LH secretory patterns also were reproducible and castrate animals demonstrated an increased pulse frequency in comparison with intact animals (Dierschke, Bhattacharya et al. 1970). These key findings, i.e. the pulsatile nature of LH secretion and the negative feedback of gonadal steroids, were subsequently reproduced in sheep (Bolt 1971). Pulsatility of LH secretion in human volunteers was described soon thereafter (Naftolin, Yen et al. 1972). LH pulses in women were

observed to follow varying frequency and amplitude, depending on the phase of menstrual cycle, with pulses observed every 1-2 hr during the early follicular phase, early luteal phase and midcycle surge, with decreased frequency (every 4 hrs) during the luteal phase (Yen, Tsai et al. 1972). Pulsatile secretion of LH has been subsequently demonstrated in a number of other mammalian species (Lincoln and Fraser 1979).

1.1.3.2 Pulsatile GnRH secretion

Direct evidence for pulsatile GnRH secretion in the primate was initially demonstrated in ovariectomised Rhesus monkeys using serial collection of hypothalamic-pituitary stalk portal blood (Carmel, Araki et al. 1976). Although correlation with peripheral LH concentrations was not possible using this model, the frequency of GnRH pulses correlated with previously documented LH pulse frequency in these animals (Carmel, Araki et al. 1976). Not long after these findings were published, pulsatile pattern of GnRH secretion with associated changes in LH concentrations was demonstrated in serial blood samples collected during pituitary surgery in patients with pituitary disease (Antunes, Carmel et al. 1978).

Meanwhile, administration of GnRH antisera was shown to abolish LH pulses (Lincoln and Fraser 1979; Caraty, Martin et al. 1984) while normal LH pulses were re-established by hourly administration of a GnRH analogue (which did not cross-react with the antiserum) (Caraty, Martin et al. 1984) –suggesting that LH pulse frequency was determined by the underlying GnRH pulse frequency. Moreover, simultaneous measurement of GnRH and LH in sheep demonstrated temporal synchrony between pulsatile secretion of the two hormones (Clarke and Cumins 1982). Simultaneous sampling of peripheral and portal blood in eugonadal, short-term castrated (1-15 days) and long-term castrated (1-6 months) rams subsequently demonstrated that GnRH was secreted in a pulsatile manner and that each GnRH pulse was associated with an LH pulse (Caraty and Locatelli 1988). Gonadal status also influenced the frequency of GnRH pulsatility with a pulse every 2-4 hours in intact rams, a pulse every 70 min in short-term castrated rams and a pulse every 36 minutes in long-term castrated rams (Caraty and Locatelli 1988).

The half-life of disappearance of exogenous GnRH from peripheral blood was initially estimated at 3.6 minutes – with elimination of most of GnRH from circulation at the time of maximal gonadotrophic stimulation (Arimura, Kastin et al. 1974). More detailed studies have shown that the half-life of GnRH (after discontinuation of an infusion) is linear for the first 8-10 min, followed by a slower component (Pimstone, Epstein et al. 1977). The half-life of the first component ranged from 5.5 to 8 min in normal subjects, 6.5-8 min in patients with impaired liver function but prolonged (12-16.5 min) in patients with renal impairment, suggesting renal metabolic clearance (Pimstone, Epstein et al. 1977).

In humans, estimation of GnRH pulse frequency therefore has to be inferred from gonadotropic output as ethical considerations preclude routine sampling of hypophyseal blood or cerebrospinal fluid. LH pulse frequency has therefore been widely used as a surrogate of GnRH pulsatility (Sauer, Frager et al. 1984; Reame, Sauder et al. 1985). Free α subunit has been proposed as a complementary marker (Whitcomb, O'Dea et al. 1990). LH, FSH, TSH and Human Chorionic Gonadotropin (HCG) are composed of two non-covalently linked subunits – alpha and beta subunits. The alpha subunit, structurally identical in all of these three gonadotropins is secreted uncombined and hence referred to as the free subunit (Corless, Bielinska et al. 1987). With a short half-life (12-15 min) and good correlation with LH, free α subunit could be used a surrogate of GnRH pulse frequency. Although there is desensitisation in free α subunit secretion at rapid GnRH frequencies, it has been shown to be a superior marker to LH at GnRH frequencies around 30 min (Hayes, McNicholl et al. 1999). However, as the alpha subunit secretion can also be modulated by perturbations in the hypothalamic – pituitary – thyroid axis, caution has to be exercised in using its pulsatility as a surrogate for GnRH pulsatility. This is particularly relevant, as factors known to modulate gonadotropin secretion can also modulate TSH secretion (Samuels and Kramer 1996). Moreover, the secretion of alpha subunit is not affected by long-term GnRH agonist therapy while LH beta subunit secretion is suppressed (Kwekkeboom, Lamberts et al. 1990), raising the possibility that alpha and beta subunit secretion may not be in tandem across physiological states.

The mechanisms by which GnRH neurons co-ordinate their activity remains to be fully elucidated (Moenter, Anthony DeFazio et al. 2003). Co-ordination between GnRH nerve fibres at the median eminence has been suggested as one possibility, as explanted rat medial basal hypothalami (MBH) demonstrate pulsatile GnRH secretion (Rasmussen 1993). Episodic multi-unit electrical activity at the MBH (adjacent to ARC) is correlated with LH release (Thiéry and Pelletier 1981; Wilson, Kesner et al. 1984), suggesting that the GnRH 'pulse generator' mechanism is anatomically located in the MBH. Not entirely inconsistent with this notion, GnRH neurons do appear to be able to create GnRH pulses on their own. GT-1 cells, a GnRH producing cell line established from the mouse hypothalamus, demonstrate regular pulsatility *in vitro* (Martínez de la Escalera, Choi et al. 1992). GnRH neurons dissected from foetal olfactory placode also demonstrated intrinsic pulsatility when cultured *in vitro* (Terasawa, Keen et al. 1999). Several other reports of similar findings in other GnRH neurons have since been reported (Maeda, Ohkura et al. 2010).

1.1.3.3 Differential Regulation of LH and FSH with varying GnRH pulsatility

The importance of GnRH pulsatility on LH and FSH secretion was first demonstrated in elegant experiments in rhesus monkeys administered GnRH as pulsatile bolus doses or continuous administration (Belchetz, Plant et al. 1978). Gonadotropin secretion was reinstated in these animals (where endogenous GnRH secretion was abolished by radio-frequency hypothalamic ablation) with hourly pulsatile administration of GnRH. In contrast, continuous infusion of GnRH only elicited a transient response. Moreover, when the animals were switched from continuous to pulsatile administration, a robust recovery of gonadotropin secretion was observed (Belchetz, Plant et al. 1978).

The stimulatory effects of GnRH on LH and FSH are not identical, with FSH demonstrating a more constitutive release (Millar, Lu et al. 2004). In ovariectomised sheep administered GnRH antisera, pulsatile secretion of LH was completely inhibited with the levels of the hormone becoming undetectable within 24 h, while the concentration of FSH fell more slowly and remained detectable (Caraty, Martin

et al. 1984). The frequency of GnRH input has been demonstrated to selectively regulate gonadotropin subunit gene transcription. Rapid GnRH pulse rates increase α and LH-β and slow GnRH pulse frequency increases FSH-β gene transcription (Dalkin, Haisenlender et al. 1989; Haisenleder, Dalkin et al. 1991; Kaiser, Jakubowiak et al. 1997). This is in keeping with earlier studies of GnRH frequency modulation demonstrating that decreasing the frequency of GnRH pulses effected a decrease in plasma LH, but an increase in FSH (Wildt, Hausler et al. 1981). Moreover, with acute progressive increases in GnRH frequency (from one pulse every 120 to 60 min, from 60 to 30 min, and from 30 to 15 min) in GnRH deficient men, mean LH rose despite a decrease in LH pulse amplitude while FSH and testosterone remained unchanged (Spratt, Finkelstein et al. 1987).

The long-half-life of FSH (274 ± 45 min in men) (Urban, Padmanabhan et al. 1991), along with cross-reactivity of some FSH assays with the α subunit makes detection of FSH pulses harder than the detection of LH pulses when serum samples are obtained from peripheral circulation. However, when samples were obtained from the hypophyseal circulation, 93% of the GnRH pulses were associated with FSH pulses (Padmanabhan, McFadden et al. 1997). Unlike LH, there is also constitutive secretion of FSH (Padmanabhan, McFadden et al. 1997).

1.1.3.4 Clinical relevance of GnRH pulsatility

1.1.3.4.1 Pubertal development

Appropriate modulation of LH pulse frequency is essential for normal development and maintenance of reproductive function. In infancy, LH secretion is pulsatile (Waldhauser, Weissenbacher et al. 1981), but later becomes quiescent. In longitudinal and cross sectional studies carried out in children aged six months to 9 years, basal and GnRH stimulated LH concentrations are higher in early childhood (< five years), subdued in mid-childhood (5-11 yrs.) and increase with pubertal development thereafter (Roth, Kelch et al. 1972; Conte, Grumbach et al. 1980). At the onset of puberty, LH pulses are synchronised with sleep (Boyar, Finkelstein et al. 1972). Reversal of sleep-wake cycle does increase day-time LH secretion during puberty, but does not eliminate nocturnal secretion (Kapen, Boar et al. 1974).

Abnormal reactivation of GnRH pulse frequency is the central pathology associated with precocious and delayed puberty (Balasubramanian, Dwyer et al. 2010).

Pre-pubertal suppression of the hypothalamus-pituitary-gonadal axis has been shown to occur in agonadal humans (Conte, Grumbach et al. 1980) and primates (Pohl, deRidder et al. 1995), suggesting that hypothalamo-hypophyseal factors play at least a part in effecting this post-natal quiescence of the reproductive axis, until puberty sets in.

1.1.3.4.2 Maintenance of reproductive function

In women, LH pulse frequency is lowest in the luteal phase, but increases during follicular and pre-ovulatory phases of the menstrual cycle (Yen, Tsai et al. 1972). In hypothalamic amenorrhoea, a clinical condition associated with anovulatory amenorrhoea and hypoestrogenemia, LH pulse frequency (and by inference GnRH pulse frequency) is lower than expected for the prevailing steroid profile and is comparable to luteal phase pulsatility in healthy volunteers (Reame, Sauder et al. 1985). LH pulse frequency in hyperprolactinemic women is also lower than that observed in healthy women in the follicular phase and increases with bromocriptine therapy (Sauer, Frager et al. 1984). Administration of opioid antagonist naloxone increases LH pulsatility in some women with hypothalamic amenorrhea and hyperprolactinemia (Khoury, Reame et al. 1987; Cook, Nippoldt et al. 1991).

In women with polycystic ovarian syndrome serum LH concentration is higher in comparison to that observed in the follicular phase of menstrual cycle (Yen, Vela et al. 1970). Subsequently, it has been demonstrated that the LH pulse frequency and amplitude were both increased in women with polycystic ovarian syndrome (Waldstreicher, Santoro et al. 1988). Moreover this increase in LH pulse frequency has also been demonstrated in both lean and obese women with polycystic ovarian syndrome (Morales, Laughlin et al. 1996; Arroyo, Laughlin et al. 1997).

1.1.3.5 Detection of LH pulsatility in peripheral blood samples

While the presence of distinct LH pulses in the peripheral circulation has been incontrovertible since it's early description by the Pittsburgh group (Dierschke,

Bhattacharya et al. 1970), a consensus is yet to be reached on the optimal approach to objectively define LH pulse frequency and amplitude in the peripheral circulation. In early reports of pulsatile LH secretion in the human setting, a rise in serum LH by 5 mIU/ml followed by a 5 mIU/ml drop sustained over two sampling points was defined as an LH pulse (Yen, Tsai et al. 1972). As the baseline LH secretion is variable across subjects, using this approach is likely to be less specific, while attempts to get independent blinded observers to count pulses is also fraught with a lack of specificity and poor reproducibility (Merriam and Wachter 1982). Therefore, baseline LH concentration was factored into the identification of LH pulses, with excursions of LH over and above 20% of local nadir defined as a pulse (Santen and Bardin 1973). While this approach was an improvement over earlier approaches, it's ability to detect pulses with a broad profile was limited and there was no physiological or functional significance attached to the 20% threshold (Merriam and Wachter 1982). This was particularly relevant as in the original study by Santen and Bardin, the apparent half-life of endogenously secreted LH in a pulse was influenced by the peak LH concentrations observed, with a significant inverse correlation between pulse peak and ensuing half-life (Santen and Bardin 1973). As with other hormones secreted in a pulsatile manner, there are two phases involved in the apparent half-life of LH. The first phase, referred to as the 'fast half-life' represents the rapid dilution of the small quantity of LH into the circulating plasma. Slow half-life represents the subsequent metabolic breakdown and excretion – apparent half-life in peripheral circulation is therefore influenced by these two factors (Santen and Bardin 1973). In conditions such as chronic renal impairment where the elimination of LH is impaired, its overall half-life is prolonged (Veldhuis, Wilkowski et al. 1993). Therefore, pulse detection based on a single excursion may miss broader pulses and the use of a fixed proportional excursion from baseline fails to recognise assay variability. Baird proposed an improvisation of the Santen and Bardin algorithm, by defining a pulse as a rise of at least four times the SD of assay sustained over two consecutive time points and also proposed the use of the mean of two preceding LH values as baseline (Baird 1978). A number of approaches were subsequently made to develop statistical tools to identify pulses but each of them had

their own relative strengths and weaknesses – the latter often outweighing the former (Merriam and Wachter 1982; Urban, Evans et al. 1988).

Development of computerised algorithms made it possible to automatically identify pulses whilst simultaneously factoring in circadian undulations in baseline LH secretion. These algorithms also incorporated assay error into the equation, whilst also being capable of identification of clustered peaks as well as identification of pulses based on both amplitude and duration (Merriam and Wachter 1982; Urban, Evans et al. 1988). The PULSAR algorithm (and its subsequent adaptations) calculated a smoothened baseline and single-point excursions from this baseline were required to have a wider amplitude than multi-point excursions (Merriam and Wachter 1982). The Cycle Detector (Clifton and Steiner 1983), on the other hand, used pre-defined thresholds to identify cyclical hormone secretion – attempting to differentiate true signals and noise. The next few years saw several new approaches being developed. Regional Dual-Threshold method used replicate values from LH immunoassays to calculate individual coefficients of variation for all samples and used pre-defined specific thresholds for upstroke and downstroke to identify pulsatile excursions from a moving baseline. Dependant on these variables, local coefficients of variation (taking a region of data at a time) were calculated automatically (Velduis, Weiss et al. 1986). Regions with high CV were therefore assigned higher thresholds for pulse detection while regions with low CV had lower thresholds (Velduis, Weiss et al. 1986).

Cluster analysis techniques, developed shortly thereafter by the same team, was designed to evaluate the behaviour or groups of data (clusters) unlike earlier approaches that considered individual data-points in isolation (Veldhuis and Johnson 1986). This approach involves the initial identification of a nadir cluster and subsequent identification of peak clusters by pooled *t* testing and marking of all significant increases (Urban, Evans et al. 1988). Once all significant increases are marked, all significant decreases in local data are also identified – a pulse being defined as a significant increase followed by a significant decrease (Veldhuis and Johnson 1986; Urban, Evans et al. 1988). The DETECT program that stemmed from efforts to study electrophoretic data was designed to be able to identify multiple

overlapping peaks and used a discrete deconvolution approach to account for apparent instantaneous secretion observed with intermittent sampling (Oerter, Guardabasso et al. 1986). The concept of deconvolution analysis for LH pulse identification was explored further by Veldhuis and colleagues by selecting pulses on the basis of the Akaike (AIC) and Bayesian information criteria (BIC) (Liu, Keenan et al. 2009). In the initial studies to detect the sensitivity and specificity of this approach, a range of minimal absolute concentration increment (0.1-0.3 IU/L) and minimal percentage peak-nadir rise (4-9%) were tested. This approach was found to have over 90% sensitivity with human, animal and simulated LH data (Liu, Keenan et al. 2009). Pulse frequency analyses described in this thesis were performed using this deconvolution method on software platforms that have been subsequently refined. Blinded data was analysed by Prof. Veldhuis at his facility at the Endocrine Research Unit at the Mayo Clinic (Rochester, MN, USA).

1.2 An introduction to kisspeptins

1.2.1 Discovery of the Kiss1 gene

KiSS1, the gene encoding kisspeptins, was first described as a suppressor of metastasis in human malignant melanoma (Lee, Miele et al. 1996). Melanoma cells (C8161) transfected with *KiSS-1* cDNA showed suppressed metastasis in comparison to control C8161 cells when injected into athymic nude mice (Lee, Miele et al. 1996). This gene was discovered in Hershey (Pennsylvania, USA) - a town founded by Milton Hershey to accommodate workers at his chocolate factory (Birchall 2009) famous for its brand of chocolates called 'Hershey's Kisses'. A number of civic amenities in Hershey are provided through funds from trusts established by the company and its founder (Birchall 2009). It is therefore not surprising that the scientists working on the gene felt it appropriate to name it after the town's famous chocolate 'Kisses'. The inclusion of SS in *KiSS1* also indicates that it is a 'suppressor sequence'.

1.2.2 Genetics of kisspeptins

KiSS1 maps to chromosome 1q32 and includes four exons of which the first two are not translated (West, Vojta et al. 1998). Exon III has 38 noncoding bases and 103

translated bases while the fourth exon has 335 translated and 121 non-translated bases (West, Vojta et al. 1998). The precursor 145 amino acid peptide is cleaved to a 54 amino acid peptide, which can be further truncated to 14, 13 and 10 amino acid C-terminal sequences, all of which share the C-terminal decapeptide sequence characterised by the Arg–Phe–NH_2 (RF Amide) C-terminal sequence (Kotani, Detheux et al. 2001).

1.2.3 Nomenclature of Kiss1 peptide and its receptor

Nomenclature of kisspeptins and their receptor has evolved rapidly and has led to multiplicity of terminology in the literature. In this thesis, the proposed nomenclature by Gottsch and colleagues (Gottsch, Clifton et al. 2009) has been adhered to, but historical nomenclature is summarized below.

1.2.3.1 Nomenclature of the peptide

As knowledge has accumulated over the years with a shift of focus for kisspeptin from cancer biology to reproductive endocrinology, nomenclature of the peptide and its receptor has evolved. To acknowledge its anti-metastatic properties, the 54 amino acid peptide product of *KiSS1* gene was christened 'metastin' (Ohtaki, Shintani et al. 2001). This 54 amino acid peptide and its shorter cleavage products with 14, 13 and 10 amino acids have since been collectively termed kisspeptins, to reflect the *KISS1* gene that encodes them. To differentiate kisspeptins of varying amino acid length, the terms kisspeptin-54, kisspeptin-14, kisspeptin-13 and kisspeptin-10 are used. Gottsch and colleagues have suggested abbreviating these to kp-10, kp-13, kp-14 and kp-54 for non-human kisspeptins with Kp-10, Kp-13, Kp-14 and Kp-54 used in the human context (Gottsch, Clifton et al. 2009).

1.2.3.2 Nomenclature of the KISS1 gene and mRNA

Lee and colleagues christened their newly discovered gene *KiSS-1* (Lee, Miele et al. 1996). The Human Genome Organization Gene Nomenclature Committee (HGNC) has proposed the symbol *KiSS1* for the gene (HGNC 2009). Incorporating these proposed names with the nomenclature guidelines and recommendations of the International Committee on Standardized Genetic Nomenclature for Mice, Gottsch

and colleagues have recommended the use of the symbol *KiSS1* for the human gene and *Kiss1* for its non-human orthologues (Gottsch, Clifton et al. 2009). *KISS1* mRNA and *Kiss1* mRNA has also been recommended as standard terms for messenger transcripts of the human and non-human genes respectively (Gottsch, Clifton et al. 2009).

1.2.3.3 Nomenclature of the kisspeptin receptor

The G–Protein Coupled Receptor (GPCR) now known to be the cognate receptor for kisspeptins was called GPR54 when it was one of the many orphan GPCRs and the gene encoding the receptor protein was also called GPR54 (Lee, Nguyen et al. 1999). The human genetic orthologues were called AXOR12 (Muir, Chamberlain et al. 2001) and hOT7T175 (Ohtaki, Shintani et al. 2001) by groups that first described them.

To simplify the number of terms used and to comply with recommendations from international committees on nomenclature of genes, it has been suggested that the symbols *KISS1R* and *Kiss1R* are used in the human and non-human context respectively to represent the gene and the protein (Gottsch, Clifton et al. 2009). Similarly, *KISS1R* mRNA and *Kiss1R* mRNA have been recommended as appropriate nomenclature for messenger transcripts from the human and non-human gene orthologues respectively (Gottsch, Clifton et al. 2009). Although these are appropriate nomenclature in the current context, naming a receptor after its ligand is fraught with the risk of adding further complexity to the literature if other ligands with affinity to the same receptor are discovered in the future. A number of authors therefore continue to use the term GPR54 to describe the kisspeptin receptor.

1.2.4 Discovery of a reproductive role for kisspeptin

Kisspeptin receptor was first described in the rat (Lee, Nguyen et al. 1999) (then called GPR54) and the human homologue (then called AXOR12 or hOT7T175) was described soon after, mapping to human chromosome 19p13.3. It has five exons, encoding a 398-amino acid protein with seven hydrophobic trans-membrane domains (Muir, Chamberlain et al. 2001) and with closest homology to the galanin receptor

family. Using RT-PCR, the receptor transcripts were shown to be abundant in placenta, pituitary, spinal cord, and pancreas (Kotani, Detheux et al. 2001).

A reproductive role for kisspeptin in humans became apparent when two groups described patients with pubertal disorders associated with mutations of its receptor (de Roux, Genin et al. 2003; Seminara, Messager et al. 2003). A number of inactivating mutations of *KISS1R* have since been reported with all phenotypes characterised by pubertal delay. Whilst there are variations in laboratory, and molecular characteristics of patients with such mutations their reproductive phenotypes are remarkably similar (Chan, Broder-Fingert et al. 2009).

An activating mutation (Arg386Pro) has also been described in a girl with precocious puberty, although the inheritance could not be characterized as the biological family was not available for genetic testing (Teles, Bianco et al. 2008). When compared to cells with wild-type transfected GPR54, cells with this mutation showed prolonged inositol phosphate accumulation and phosphorylation of extracellular signal–regulated kinase, suggesting extended activation of intracellular signaling by the mutant GPR54 (Teles, Bianco et al. 2008).

Missense mutations have also been reported in the *KISS1* gene in three unrelated children with central precocious puberty (Silveira, Noel et al. 2010). Functional characterization studies of these mutant peptides demonstrated higher resistance to *in vitro* degradation but normal affinity at the kisspeptin receptor – thus suggestive of increased bioavailability as the mechanism by which these abnormal kisspeptin peptides effect precocious puberty (Silveira, Noel et al. 2010).

1.2.5 Stimulatory effect of Kisspeptin on GnRH and gonadotropin secretion

The stimulatory effects of kisspeptin on LH secretion have been documented in a number of species. In adult male mice, 1 nmol of both kp-10 and kp-54 delivered intracerebroventricularly (ICV) stimulated significant LH release as estimated by orbital blood sampling 30 min post kisspeptin (Gottsch, Cunningham et al. 2004). ICV administration of varying doses of kp-54 (1 fmol to 5 nmol) in mice showed a significant increase in 30 min serum LH in comparison to vehicle with doses as low as 1 fmol (Gottsch, Cunningham et al. 2004).

LH response to kisspeptin-54 is prevented in mice pre-treated with a potent GnRH antagonist, acyline (Gottsch, Cunningham et al. 2004), suggesting that the stimulatory effect of kisspeptin on LH secretion is mediated through GnRH. Comparison of venacaval serum LH responses to intraperitoneal injections of mouse kisspeptin 105-119 in wild-type and *Kiss1R* knockout mice have shown a significant rise in LH in the wild-type and no detectable response in the mutants (Messager, Chatzidaki et al. 2005), suggesting that the stimulation of gonadotropins by kisspeptin is mediated through *Kiss1R*.

These findings have also been replicated in larger species. In prepubertal gilts, ICV and intravenous (IV) administration of 10 μg and 100 μg of kisspeptin (peptide length unclear from published manuscript) showed a dose dependent increase in serum LH, as estimated through multiple small volume sampling through indwelling jugular cannulae (Lents, Heidorn et al. 2008). Administration of 380 pM of kp-10 IV in ovariectomised sheep have shown a doubling of mean serum LH and LH pulse amplitudes with significant increase in area under the curve (AUC) of LH pulses (Arreguin-Arevalo, Lents et al. 2007). The AUC of LH following 14 pM GnRH was higher than the AUC following 380 pM of kp-10 (Arreguin-Arevalo, Lents et al. 2007). Passive immunization with anti-GnRH antibody abolished these stimulatory effects of kisspeptin (Arreguin-Arevalo, Lents et al. 2007), further suggesting that kisspeptin stimulation is mediated through GnRH receptor activation. ICV administration of Kp-10 in sheep has also shown significant increase in serum LH, with tandem assays of GnRH from intracerebroventricular catheters and LH in peripheral circulation showing temporally correlated increases in GnRH and LH during kisspeptin infusion (Messager, Chatzidaki et al. 2005), suggesting stimulation of GnRH secretion by kisspeptin as its mode of gonadotropin stimulation. LH response (AUC, when compared to vehicle) to intravenous kp-10 also depends on the reproductive status of the ewe, with significantly higher response in the non-breeding season than in the breading season (Smith, Saleh et al. 2009). Within the breeding season, the highest LH response in ewes was observed in the late follicular phase (Smith, Saleh et al. 2009). In anestrous sheep, intravenous administration of doses as low as 6 nmol Kp-10 robustly elevate plasma LH and FSH with human Kp-10 and murine kp-10 showing equipotency (Caraty, Smith et al. 2007). By simultaneous

sampling of CSF GnRH and serum LH in experimental sheep during an infusion of Kp-10 (50 nmols over four hours), simultaneous increases in LH and FSH has also been documented, providing further evidence of kisspeptin-related increases in gonadotropin secretion being mediated by GnRH (Messager, Chatzidaki et al. 2005).

In prepubertal Holstein heifers, injection of 1 mg of Kp-10 (mean dosage 5 μg/kg) effected a five-fold increase in LH from baseline with peak LH noted at a mean of 27 minutes from baseline (Kadokawa, Matsui et al. 2008). This study also noted a significant rise in growth hormone (GH) from baseline with peak serum concentrations noted at a mean of 75 minutes post kisspeptin administration (Kadokawa, Matsui et al. 2008). However, this stimulatory effect of kisspeptin on pituitary somatotroph cells remains to be reproduced.

In male rhesus monkeys primed with pulsatile GnRH, intracerebroventricular and intravenous administration of 100 μg of Kp-10 (approximately 30 μg/kg) increased plasma LH over 25-fold from baseline (Shahab, Mastronardi et al. 2005). Pre-treatment with GnRH antagonist acyline abolished these responses to kisspeptin, further suggesting that the modulation of gonadotropins by kisspeptin is mediated through GnRH signaling (Shahab, Mastronardi et al. 2005).

1.2.5.1 Comparison of stimulatory efficacy of various kisspeptin fragments

Comparison of 60-min serum samples of LH and testosterone in male Wistar rats following acute subcutaneous injections of a wide range of doses (0.1, 0.3, 1.0, and 50 nmol) of Kp-10, Kp-14 and Kp-54 showed a significant increase only in the Kp-54 treated animals at doses of 1 and 50 nmol (Thompson, Murphy et al. 2006). Murine kp-52 and human Kp-10 have been shown to effect a rise in LH at 30 minutes post-injection when administered ICV to adult male mice with the longer peptide effecting higher absolute LH, but statistically similar to serum LH post kp-52 injection (Gottsch, Cunningham et al. 2004). When 3.0 nmol/kg each of kp-10 and kp-52 were administered intravenously to male rats, similar peak LH levels achieved (Tovar, Vazquez et al. 2006). Four boluses of Kisspeptin-10 administered at 75-min intervals evoked robust LH secretory bursts with similar pulse size. Serum LH

responses to intravenous kp-52 were slightly greater in magnitude and duration (Tovar, Vazquez et al. 2006).

Mikkelsen and colleagues have studied the acute effects of murine kp-10, murine kp-52, human Kp-54 and human Kp-10 on serum testosterone in experimental mice (Mikkelsen, Bentsen et al. 2009). Adult male NMRI mice receiving 3nmol/kg or more of all four kisspeptin fragments resulted in similar elevation in serum free testosterone. Maximal effect was seen at 10 nmol/kg, with the higher dose of 30 nmol/kg achieving similar elevations of serum testosterone. Dose response curves observed with all the four peptides were similar except for Kp-54 (human) showing non-significantly higher potency. The ED50 was estimated as 3nmol/kg.

The time-course of acute stimulatory effects of various kisspeptin fragments has also been studied (Mikkelsen, Bentsen et al. 2009). Murine kp-10, murine kp-52, human Kp-54 and human kp-10 all maximally elevated serum testosterone at 30 minutes following intraperitoneal injection of 10 nmol/kg of each peptide in adult male NMRI mice. Shorter peptides show quicker onset of action within the first 10 minutes while Kp-54 showed a sustained stimulatory effect on serum testosterone lasting beyond 60 minutes (Mikkelsen, Bentsen et al. 2009).

1.2.5.2 Stimulatory effect of kisspeptin in healthy human subjects

Kisspeptin-54, when administered intravenously or subcutaneously in healthy men and women results in robust and significant increase in serum LH and gonadal steroids (Dhillo, Chaudhri et al. 2005; Dhillo, Chaudhri et al. 2007).

In a study designed to establish dose response curve for Kp-54, healthy male volunteers were infused intravenously at doses ranging from 0.125 to 40 pmol/kg/min for 30 minutes with a further 60 minutes of intravenous infusion at half the initial rate (Dhillo, Chaudhri et al. 2005). Mean LH increased in a dose dose-depended manner from 0.25pmol/kg/min to 12 pmol/kg/min. Mean LH for doses above 12 pmol/kg/min tended to be non-significantly lower than the value achieved with 12 pmol/kg/min.

The time course of LH and FSH responses to an intravenous infusion of 4 pmol/kg/min of Kp-54 for 30 minutes (i.e., 240pmol/kg), followed by half this rate for a further 60 min has also been demonstrated in 6 male volunteers (Dhillo, Chaudhri et al. 2005). Following discontinuation of the infusion, plasma kisspeptin immuno-reactivity (Kp-IR) decreased with first order kinetics with a calculated half-life of 27.6 +-1.1 min. Peak LH levels correlated with Kp-IR and returned to baseline 3 hours following the discontinuation of kisspeptin infusion. However, after an initial drop mirroring LH, serum testosterone showed a sustained rise, with peak values achieved 24 hours after the start of the infusion. However, the significance of this pattern of change in serum testosterone is uncertain as the control arm also showed a similar profile.

Kp-54 (0 to 6.4 nmol/kg doses) administered subcutaneously (in the abdomen) in healthy women has also been shown to result in dose-dependent increases in LH, and FSH but not free estrogen index (Dhillo, Chaudhri et al. 2007). When 0.4 nmol/kg of Kp-54 was administered subcutaneously as bolus doses in women during follicular, pre-ovulatory and luteal phases of their menstrual cycles (Dhillo, Chaudhri et al. 2007), maximal stimulation of LH was seen in the pre-ovulatory phase (Dhillo, Chaudhri et al. 2007), suggesting variation in kisspeptin dependent LH release is likely to be effected at the level of the pituitary. In studies involving the administration of an identical dose of GnRH to women across the menstrual cycle, maximal sensitivity to GnRH has been demonstrated in the pre-ovulatory phase (Yen, VandenBerg et al. 1972). Therefore, it is plausible that kisspeptinergic stimulation of GnRH secretion could be constant irrespective of the prevailing steroid milieu but that the amplified LH response observed in the pre-ovulatory phase is mediated by increased pituitary sensitivity to GnRH.

1.2.5.3 Effect of chronic or continuous Kisspeptin administration on gonadotropin secretion

Continuous administration of exogenous GnRH desensitizes the HPG axis, suppressing gonadotropin release from the pituitary following an initial stimulatory effect (Belchetz, Plant et al. 1978). Therefore, efforts have been made to assess the impact of continuous infusions of kisspeptin in a number of experimental settings.

In adult male Wistar rats weighing 275–325g, continuous subcutaneous administration of 50 nmol/day of Kp-54 effected a three-fold increase in LH on day one, but this stimulatory effect on LH was lost after 2 days with vehicle and treatment arms showing similar LH profiles on day three (Thompson, Murphy et al. 2006). In parallel with the LH excursion, testosterone also increased, with a four-fold increase was seen in plasma free testosterone on day one. This stimulatory effect on LH was also lost after 2 days (Thompson, Murphy et al. 2006).

In experimental sheep infused with Kp-10 over four hours, LH levels were robustly elevated, but serum LH declined from its peak values before the end of the infusion while CSF GnRH remained elevated for 2 hours following the discontinuation of the infusion (Messager, Chatzidaki et al. 2005). This raises the possibility that desensitization to GnRH could be occurring at the level of the pituitary gonadotrope.

In agonadal juvenile male monkeys primed with GnRH, continuous intravenous infusion of 100μg/hr of Kp-10 led to an initial stimulatory effect on serum LH concentrations (Seminara, DiPietro et al. 2006) followed by a decrease in serum LH, dropping to levels comparable to vehicle infusion by 12 hours. Whilst maintained on the infusion, these experimental animals continued to respond to GnRH and N-methyl-DL-aspartic acid (NMDA) (10 mg/kg) but not to intravenous bolus kisspeptin (10 μg Kp-10) suggesting that the pituitary responsiveness to GnRH is preserved whilst kisspeptinergic stimulation has been desensitized. Less than a day after discontinuing the infusion, the ability of an intravenous bolus of 10 μg of Kp-10 to stimulate LH was re-established.

In adult male rhesus monkeys infused continuously with 200 μg/hr and 400 μg/hr (15 and 30 nmol/kg•h) of Kp-10, serum LH returned to pre-infusion baseline after 24 hours of infusion following an initial robust surge (Ramaswamy, Seminara et al. 2007). LH responses to NMDA and GnRH on the first day of the study were comparable with vehicle infused control animals on day four of the low dose infusion. However, animals on the higher dose infusion (400 μg/hr) showed a reduced response. LH responses to bolus doses of intravenous Kp-10 were diminished whilst the animals were being infused with Kp-10. These effects recovered fully in the low-dose group and partially in the higher dose group.

Reduction in LH pulse frequency (four pulses per 6 h to less than one pulse per 6 h) and LH pulse amplitude were noted in the high dose group but not in the low dose cohort. Despite a reduction in circulating LH in animals infused with high dose Kp-10, serum testosterone was significantly higher than baseline and control animal, prompting the authors to suggest a direct effect of Kp-10 at the level of the testes.

In women with hypothalamic amenorrhea receiving subcutaneous Kp-54 (37 μg/kg; 6.4 nmol/kg) twice daily for two weeks, potent increases in serum LH and FSH seen on day one were significantly diminished at the end of the study (Jayasena, Nijher et al. 2009). No changes in LH pulsatility were observed in this study.

1.2.6 Neuroanatomy of the kisspeptin neuronal network

1.2.6.1 Human hypothalamus

Initial studies on the neuroanatomical distribution of kisspeptin neurons in the human brain were carried out in autopsy samples from premenopausal women (n=8; age = 32 ± 2.7 years; age range, 21–41 years) and postmenopausal women (n=9; age = 72 ± 2.7 years; range, 59–86 years) were analysed with hybridisation histochemistry. Endometrial biopsies were obtained to confirm reproductive status. The only focus of *KISS1* expression was limited to the infundibular nucleus (homologous to the ARC in lower species), with no *KISS1* expression in the preoptic area homologous to rodent AVPV. *KISS-1 mRNA* expressing neurons in the infundibular nucleus were more than double in the postmenopausal sections, suggesting a negative feedback for sex steroids on kisspeptin neurons (Rometo, Krajewski et al. 2007).

A more recent study, carried out using both male and female autopsy samples, have demonstrated the presence of kisspeptin cell bodies in the infundibular nucleus, in keeping with earlier human and non-human studies (Hrabovszky, Ciofi et al. 2010). The study also showed another kisspeptin cell population in the rostral periventricular zone in female hypothalami. Female hypothalami also demonstrated higher kisspeptin immunoreactivity in the infudibular nucleus, raising the hypothesis that kisspeptin neurons are sexually dimorphic in the human. Axo-somatic, axo-dendritic and axo-axonal contacts between kisspeptin- and GnRH-immunoreactive axons were also demonstrated in the infundibular stalk (Hrabovszky, Ciofi et al.

2010) in keeping with data obtained from primates (Ramaswamy, Guerriero et al. 2008). Three-quarters of kisspeptin-immunoreactive cells in the infundibular nucleus were also shown to co-express neurokinin B (Hrabovszky, Ciofi et al. 2010), in keeping with observations in sheep (Goodman, Lehman et al. 2007) and rodents (Navarro, Castellano et al. 2011). As the population (Crowley and Mcarthur 1980) of Arcuate/Infundibular kisspeptin neurons co-localise neurokinin B and dynorphin, these neurons are also referred to as KNDy (Kisspeptin - Neurokinin B – Dynorphin) neurons (Lehman, Coolen et al. 2010).

1.2.6.2 Non-human primates

Experimental studies on reproduction in non-human primates offer valuable insights into human physiology as these animals also undergo distinct pubertal maturation associated with a GnRH surge (Shahab, Mastronardi et al. 2005). As this species is also similar to the human in terms of having a distinct reproductive cycle with menstruation in women, primates are considered to be a better model of reproductive function than rodents (Pimstone, Epstein et al. 1977). However, where juvenile primates are used, the direct translation to adult human function is difficult, as various developmental and endocrine factors might confound observations. In particular, as these animals do not yet have a GnRH pulse generator with pulse frequency, and pulse-amplitude similar to adult animals, GnRH is administered as discrete fixed pulses(Seminara, DiPietro et al. 2006). Whilst this model has inherent strengths in mechanistic studies of hierarchical reproductive function, extrapolation of their findings to diseases associated with altered GnRH/LH pulsatility has to be tempered with an understanding of such limitations.

Shahab and colleagues localised Kiss1 expression to the medial ARC nucleus using coronal sections from ovariectomised adult rhesus monkeys treated with estradiol (Shahab, Mastronardi et al. 2005). In the same study, Kiss1R mRNA expression was localised to the medial and lateral aspects of the ARC nucleus (Shahab, Mastronardi et al. 2005). The presence of focal Kiss1 expression has also been documented in the medio-basal hypothalamus (MBH, an area of the hypothalamus including the ARC) in agonadal male rhesus monkeys (Shibata, Friedman et al. 2007). Testosterone replacement with an LH suppressive dose using testosterone-filled silastic capsules

led to a decrease in Kiss1 mRNA in the MBH of these animals (Shibata, Friedman et al. 2007). The localisation of Kiss1 mRNA in the ARC nucleus has also been documented in both ovariectomised and non-ovariectomised adult cynomolgus monkeys, with the former showing significantly higher Kiss1 expression (Rometo, Krajewski et al. 2007). None of these studies showed demonstrable Kiss1 mRNA in the preoptic area (POA) of the hypothalamus.

1.2.6.3 Rodents

In rodents, two distinct dense populations of kisspeptin neurons exist; one in the rostral continuum of cells abutting the third ventricle in the preoptic periventricular nuclei (including the anteroventral periventricular nucleus [AVPV], the periventricular nucleus [PeN]) and the arcuate (ARC) nucleus) (Clarkson and Herbison 2006). The continuum of periventricular area extending caudally from the level of the organum vasculosum of the lamina terminalis to the AVPV and PeN has been recently described as the rostral periventricular region of the third ventricle (RP3V) (Herbison 2008; Clarkson, Tassigny et al. 2009). These periventricular nuclei show marked sexual dimorphism with the adult female hypothalami characterized by 10-fold greater number of kisspeptin neurons (Clarkson and Herbison 2006). In addition to the two dense populations of cell bodies in the RP3V and ARC, kisspeptin immunoreactive cells or Kiss1 mRNA have been described in the brainstem, dorsomedial nucleus, amygdala, posterior hypothalamus and the striae terminalis in mice. However, immunocytochemical analysis with more specific antibodies to mouse kisspeptin has only confirmed the presence of low-density kisspeptin immunoreactive cells in the posterior hypothalamus and the dorsomedial nucleus.

Kisspeptin fiber distribution matches the distribution of cell bodies, showing predominant concentration in the ARC with dorsal and lateral extensions to adjacent regions of the brain (Clarkson and Herbison 2006). Kisspeptin immunoreactive cell bodies are undetectable in the AVPV and PeN in the postnatal juvenile period (P10) in both male and female mice but increase many-fold through pre-pubertal (P25) and peripubertal (P35) stages of reproductive development. In the male, cell bodies in both AVPV and PeN reach their peak quantity by puberty while in the female, there

is a doubling of cell bodies in the AVPV post puberty (Clarkson and Herbison 2006). The post-natal development of cell bodies in the ARC is yet to be established, although the distribution of kisspeptin immunoreactive fibers has been observed at all ages (Clarkson and Herbison 2006).

Normal female mice express significantly more Kiss mRNA in the AVPV than normal male mice under identical hormonal milieu, when analyzed by in situ hybridisation. This difference also appears to occur perinatally, as neonatally androgenised female mice show a Kiss mRNA distribution similar to male mice (Kauffman, Gottsch et al. 2007). There is no sexual dimorphism in Kiss mRNA expression in the ARC nucleus (Kauffman, Gottsch et al. 2007) in mice. The distribution of kisspeptin immunoreactive cell bodies and fibres in mice has also been mapped by Clarkson and colleagues (Clarkson, Tassigny et al. 2009).

Using transgenic Kiss1R LacZ knock-in mouse model, abundant Kiss1R expression was noted in a number of brain regions. Within the hypothalamus, Kiss1R expressing cells were found primarily in the GnRH neurons and a cell population in the posterior periventricular hypothalamus. Moreover, less than 40% of GnRH neurons in these mice had Xgal Kiss1R staining at birth, compared with 60-70% of peripubertal and adult GnRH neurons (Herbison, d'Anglemont de Tassigny et al. 2009). This suggests post-natal maturation of the Kiss1R system in the GnRH neuron.

1.2.6.4 Sheep

As with rodents, kisspeptin cell body immunoreactivity has been identified in the ARC nuclei of sheep brain using immunocytochemistry (Smith, Coolen et al. 2008). Immunocytochemical analysis of ovine hypothalamus with antiserum specific to ovine kp-10 has demonstrated the presence of dense kisspeptin immunoreactive neurons in the ARC nucleus (Franceschini, Lomet et al. 2006) confirming similar findings obtained using rabbit polyclonal antibody against kp-10 (Pompolo, Pereira et al. 2006). In situ hybridisation has confirmed the presence of the largest population of *kiss1 mRNA* expressing cells in the ovine hypothalamus to be located in the ARC (Smith, Clay et al. 2007). A significant elevation of *kiss1 mRNA* has also been

demonstrated in the ARC prior to the onset of ovine breeding season, suggesting a role of kisspeptin as a neuroendocrine reproductive trigger (Smith, Clay et al. 2007). *Kiss1 mRNA* expression in the ARC is also under negative feedback from E and P (Smith, Clay et al. 2007). Kisspeptin neurons in the ovine ARC also co-express dynorphin-A and neurokinin-B, with the former co-expressed in almost all kisspeptin immunoreactive neurons and the latter in over two-thirds (Goodman, Lehman et al. 2007). The expression of *kiss1 mRNA* (Estrada, Clay et al. 2006; Smith, Li et al. 2009) and the number of kisspeptin immunoreactive cells (Smith, Li et al. 2009) are up-regulated in the caudal ARC during the preovulatory period of the ewe oestrous cycle suggesting that the ARC plays a role in mediating pre-ovulatory GnRH surge.

The population of kisspeptin immunoreactive neurons putatively corresponding to the R3PV periventricular nuclei is more laterally located within the pre-optic area in sheep (Franceschini, Lomet et al. 2006; Pompolo, Pereira et al. 2006; Smith, Coolen et al. 2008). This population of kisspeptin neurons is located in the medial preoptic area, a region which in the ovine hypothalamus known to have abundant GnRH neuronal population (Franceschini, Lomet et al. 2006). Studies using ovine kp-10 antisera have shown a higher quantity of kisspeptin immunoreactive cells (Franceschini, Lomet et al. 2006) in the preoptic area in comparison to immunocytochemical analysis using human kp-10 antibodies (Pompolo, Pereira et al. 2006), suggesting a differential sensitivity of kisspeptin neurons to various kisspeptin antisera. *In situ* hybridisation complements immunohistochemcal studies by confirming the presence of *kiss1 mRNA* expression in the pre-optic area (Smith, Clay et al. 2007). No changes in the number of kisspeptin immunoreactive cell bodies was observed between non-breeding and breeding seasons (Smith, Coolen et al. 2008). *Kiss1* mRNA expression is higher in the late follicular stage (preceding GnRH/LH surge) when compared to late luteal phase (Smith, Li et al. 2009). Kisspeptin protein and *kiss1 mRNA* expression in the pre-optic area is increased with estradiol treatment in ovariectomised ewes (Smith, Li et al. 2009) although only 50% of the kisspeptin immunoreactive cells in this area co-express ER alpha (Franceschini, Lomet et al. 2006). Dynorphin A and neurokinin B co-expression is also not seen in this ovine population of kisspeptin neurons (Goodman, Lehman et al. 2007).

1.2.7 Kisspeptin analogues.

As the central role played by the kisspeptin-GPR54 system in the development and maintenance of normal adult reproductive function has become apparent, efforts have been made to make analogues to explore the physiological roles and potential pharmacological applications in modulating the HPG axis. Roseweir and co-workers have systematically shortened and substituted amino acids in the kisspeptin-10 decapeptide sequence to develop potent antagonists (Roseweir, Kauffman et al. 2009; Pineda, Garcia-Galiano et al. 2010). Results from these studies and their relevance in understanding the physiological regulation of reproductive function are reviewed elsewhere in detail (Millar, Roseweir et al. 2010). Using similar methodical substitution of amino acid sequence, analogues with higher *in vitro* binding affinity and increased in *vivo* potency and efficacy have also been reported (Curtis, Cooke et al. 2009).

In brief, kisspeptin antagonists have been demonstrated to show

- Inhibition of kisspeptin stimulation of GnRH firing in brain slices from genetically modified female mice with green fluorescent protein expression in the GnRH.
- Inhibition of pulsatile GnRH release in pubertal female rhesus monkeys.
- Inhibition of post-castration LH rises in rats and mice.
- Inhibition of kisspeptin-stimulated LH secretion in intact and castrated rats.
- Inhibition of LH pulsatility in ovariectomised sheep.
- Inhibition of pubertal development in rodents with decrease in vaginal opening, decreased uterine and ovarian size.
- Inhibition of pre-ovulatory LH surge in the female rat.

Unpublished data have shown marked suppression of gonadotropins with continuous administration of such potent analogues (Ohkura, Tanaka et al. 2011). Early phase clinical trials are currently underway to explore the therapeutic value of this approach in prostate cancer (Takeda.com, accessed 13 Sept 2011).

1.3 Regulation of GnRH and gonadotropin secretion

Development and maintenance of normal adult reproductive function requires a coordinated interplay between environmental, metabolic and environmental factors. The GnRH-gonadotrope system plays a central role in the regulation of reproduction by being the final integrator of such diverse signals.

1.3.1 Sex steroid feedback

A crucial role for sex steroids in the feedback regulation of GnRH neurons and/or gonadotropes in the human was initially proposed as serial blood sampling and gonadotropin assays in apparently healthy women through phases of menstrual cycle showed an uneven distribution. A clear mid-cycle surge evident in LH and FSH concentrations (Midgley and Jaffe 1971). Two distinct mechanisms were proposed as plausible to mediate this effect. First, that the GnRH secretory pattern is altered in response to the steroid milieu. Second, that the sensitivity of the gonadotropes to a given GnRH input is sex-steroid dependant (Midgley and Jaffe 1971). Despite focussed and intense effort by a number of groups, the relative contribution of these two modes of feedback remains controversial, partly due to inter-species differences (Midgley and Jaffe 1971).

Hypothalamic secretion of GnRH increases during proesterus in rats (Levine, Bauer-Dantoin et al. 1991) sheep (Clarke 1988) and non-human primates (Pau, Berria et al. 1993). However, in Rhesus monkeys with hypothalamic lesions induced to eliminate GnRH secretion, normal ovulation was restored by exogenous GnRH administered as a pulse every hour throughout the cycle, prompting the investigators to conclude that it was the “ebb and flow” of estrogen feedback on the pituitary that effected the LH surge (Knobil, Plant et al. 1980). In the human setting, induction of ovulation in patients with Kallmann syndrome with pulsatile GnRH delivered as 25 ng/kg pulses every 2 hours over 27 days provide further support to this hypothesis (Crowley and Mcarthur 1980). Moreover, there is evidence to suggest that in the human context, endogenous GnRH secretion is potentially diminished during the pre-ovulatory LH surge (Hall, Taylor et al. 1994). In studies carried out in healthy women across various phases of the menstrual cycle, there was greater suppression of gonadotropins with lower doses of a competitive GnRH receptor antagonist during

the mid-cycle surge, in comparison to the other phases of the menstrual cycle (Hall, Taylor et al. 1994). Whilst these findings support the notion that pituitary sensitivity to GnRH is enhanced during the mid-cycle surge, it is also consistent with modulation of GnRH secretion across the menstrual cycle (albeit a decrease in GnRH secretion and not an increase in GnRH feedback as expected) (Plant 2008). When men with hypogonadotropic hypogonadism treated with pulsatile GnRH therapy were administered exogenous estradiol or testosterone, gonadotropin concentrations decreased, demonstrating inhibitory effects of these hormones at the pituitary (Bagatell, Dahl et al. 1994). A direct effect of estrogen on gonadotropes is further demonstrated by the *in vitro* inhibition of LH secretion from rat pituitary gonadotropes in culture (Emons, Ortmann et al. 1986). Moreover, LH and FSH levels as well as LH pulse amplitude have been demonstrated to be decreased in healthy volunteers and GnRH-deficient men on long-term pulsatile GnRH therapy exposed to exogenous E2 (Finkelstein, O'DEA et al. 1991). However, a number of studies, as discussed above, have demonstrated an inhibitory role for sex steroids at the hypothalamus - thus suggesting that there is feedback regulation occurring at the level of both the pituitary and the hypothalamus. Recent experimental evidence also supports this notion of dual-site feedback (Pitteloud, Dwyer et al. 2008), as anastrazole-suppressed healthy male volunteers demonstrated increases in LH pulse frequency and amplitude despite GnRH secretion remaining unaffected (Hayes, Seminara et al. 2000).

1.3.1.1 Estrogen feedback

A biphasic effect of estradiol on gonadotropin secretion has long been established, with an initial negative feedback (with greater suppression of FSH) and a subsequent positive feedback that is more prominent for LH (Yen and Tsai 1971; Keye and Jaffe 1975). Pulse frequency analyses have since demonstrated a decrease in pulse frequency within 24 hours of exposure to exogenous estradiol, followed by an increase (observed at days 5 and 10 in the study) and a decrease thereafter (Veldhuis, Evans et al. 1987). These observations in human volunteers are consistent with studies in non-human primates where estradiol administration was shown to inhibit GnRH pulse generator Multi-Unit Electrical Activity (Kesner, Wilson et al. 1987) as

well as studies in rodents demonstrating that insertion of estrogen micro-implants in the ARC nucleus results in diminished LH pulse frequency (Akema, Tadokoro et al. 1984). When the anti-estrogen clomiphene citrate was administered to women in early follicular phase, LH and FSH increased with associated increase in LH pulse frequency, suggesting a suppressive role for estrogen at the supra-pituitary level (Kerin, Liu et al. 1985). Similar observations have also been in made in men – with decreased LH pulse frequency and amplitude observed during administration of estradiol and converse enhancement of these markers when tamoxifen was administered (Veldhuis and Dufau 1987).

Molecular mechanisms underpinning estrogen feedback remain to be fully elucidated. Immunohistochemical studies have failed to demonstrate the presence of estrogen receptor α (ERα) on GnRH neurons (Herbison 1998). Although some GnRH neurons express ERβ, these receptors may not have a functional role in effecting steroid feedback as ERβ knockout mice demonstrate normal fertility (Lubahn, Moyer et al. 1993; Krege, Hodgin et al. 1998). Moreover, the rapid onset of inhibitory effects of estradiol on LH pulse frequency would suggest that non-genomic mechanisms not involving classic nuclear receptor pathway might also be involved (McDevitt, Glidewell-Kenney et al. 2008). Membrane receptors of estrogen located on GnRH neurons (GPR30) have also recently been described, suggesting that estrogen feedback on GnRH neurons is multimodal (Noel, Keen et al. 2009; Sun, Chu et al. 2010).

There is now a wealth of evidence to suggest that kisspeptin neurons in the ARC mediate estrogen feedback on GnRH neurons in the female mouse and rat (Oakley, Clifton et al. 2009). In postmenopausal women, the infundibular nucleus (the homologous location to ARC in the human hypothalamus) is characterised by an increase in the size and number of *KISS1* expressing neurons (Rometo, Krajewski et al. 2007). Similar findings were also observed in ovariectomised cynomolgus monkeys (Rometo, Krajewski et al. 2007). Moreover, in these non-human primates, estrogen (or estrogen + progesterone) add-back has been demonstrated to be associated with a decrease in the number of *kiss1* neurons (Rometo, Krajewski et al. 2007). These findings are also consistent with increases in kiss1, GPR54 and GnRH-

1 mRNA expression observed in the medial basal hypothalamus of postmenopausal macaques in comparison to eugonadal adults (Kim, Jessen et al. 2009). Prolonged estrogen deprivation in rhesus monkeys ovariectomised four years prior to experimentation was associated with increased KiSS1 and NKB expression, with estradiol administration reversing these findings (Eghlidi, Haley et al. 2010). Shorter duration since ovariectomy and fluctuations in circulating estradiol levels was, however, not associated with changes in KiSS-1, NKB or PDYN (prodynorphin) expression in rhesus monkeys (Eghlidi, Haley et al. 2010; Smith, Shahab et al. 2010).

The presence of two distinct kisspeptin populations (in the ARC nucleus and rostral periventricular region of the third ventricle (RP3V) in laboratory rodents with different functional roles makes the interpretation of findings from rodent and sheep models more complex. Nevertheless, neurophysiological observations pertaining to estrogen feedback at the ARC in these species, are consistent with observations in the primate (Oakley, Clifton et al. 2009). Studies in ERα and ERβ knock-out mice have demonstrated that modulation of KiSS1-mRNA expression by estradiol is signalled through ERα and that almost all kisspeptin neurons co-express ERα (Smith, Cunningham et al. 2005). Non-classic signalling through ERE-independent pathways have also been described in the kisspeptin rodent ARC (Gottsch, Navarro et al. 2009). Studies in sheep have also demonstrated normalisation of increased KiSS1-mRNA expression in ovariectomised ewes by add-back of estradiol (Smith, Clay et al. 2007).

1.3.1.2 Testosterone feedback

Early studies of testosterone feedback on gonadotropin secretion demonstrated that LH and FSH pulse frequency are enhanced in hypogonadal men and that exogenous testosterone decreases these parameters, suggesting that testosterone (and/or its metabolites have an inhibitory effect on hypothalamic GnRH secretion (Matsumoto and Bremner 1984). These findings are in keeping with similar observations made in rodents (Steiner, Bremner et al. 1982), sheep (D'Occhio, Schanbacher et al. 1982) and non-human primates (Plant 1982).

As testosterone is aromatised to estradiol, whether testosterone effected its inhibitory effect on LH secretion directly or through conversion to estradiol had to be established. Similarly as 5α-reductase converts testosterone to dihydrotestosterone, (DHT, a non-aromatisable androgen), differentiation of effects of testosterone and DHT are also important. Santen's elegant experiments involving the infusion of estradiol, testosterone and dihydrotestosterone to healthy men demonstrated that the effects of DHT were comparable to that of testosterone (Santen 1975). Moreover, GnRH sensitivity was preserved in men infused testosterone, whilst this was diminished in those infused estradiol (Santen 1975). Absence of inhibitory effects when DHT was administered to men with hypogonadotropic hypogonadism (IHH) treated with pulsatile LH therapy further suggests that DHT does not play a role, at least at the level of the pituitary (Bagatell, Dahl et al. 1994). Moreover, in healthy men pre-treated with ketoconazole (achieving biochemical castration through desmolase and aromatase inhibition), replacement therapy with physiological doses of testosterone decreased LH pulse frequency, suggesting a direct action of testosterone at the hypothalamus (Pitteloud, Dwyer et al. 2008). However, LH pulse frequency and amplitude have been demonstrated to remain unchanged in IHH men on GnRH therapy (with fixed doses and frequency) exposed to identical intervention, suggesting that the effects of testosterone on gonadotropes, would require aromatisation (Pitteloud, Dwyer et al. 2008).

Steroid receptor cross-talk between AR and ER is another emerging paradigm that makes it harder to differentiate the functional roles of androgen and estrogen. Although these effects could be specific to the tissue and development stage of animal models studied, down-regulation of AR expression has been described in the prostate following neonatal estrogen exposure (Woodham, Birch et al. 2003) and co-transfection of AR and ER to cell lines modulate AR transcription (Kumar, Leo et al. 1994). Similarly, co-administration of DHT and estradiol decreased porcine endometrium ERα and ERβ mRNA in comparison to estradiol alone (Cárdenas and Pope 2004).

Double-label immunohistochemistry studies in ovine hypothalami have demonstrated that few GnRH neurons (less than 0.5%) express AR (Herbison, Skinner et al. 1996).

GnRH neurons are thus considered to be reliant on intermediary neuronal populations for mediating testosterone feedback. Experimentation in male animal models suggests a key role for KNDy neurons in the ARC in effecting such feedback as these neurons express AR. Suppression of gonadotropin secretion using testosterone implants in male rhesus monkey is associated with a reduction of *Kiss1* mRNA in the medial basal hypothalamus (Shibata, Friedman et al. 2007), consistent with similar findings observed in rodents (Irwig, Fraley et al. 2004; Navarro, Castellano et al. 2004). Testosterone induced suppression of *Kiss1* mRNA in the rodent ARC is identical to that observed with estradiol, but more than that observed with DHT administration, suggesting that aromatisation of testosterone is required to effect at least part of its effect on KNDy neurons (Smith, Dungan et al. 2005). Moreover, post-orchidectomy rise in LH in rodents can be blocked by kisspeptin antagonists in male rodents, further suggesting that kisspeptin-GPR54 system mediates hypothalamic androgen feedback (Roseweir, Kauffman et al. 2009).

1.3.1.3 Progesterone feedback

It has long been established that exogenous progesterone administration reduces LH pulse frequency, enhances LH pulse amplitude and decreases mean LH in women in the follicular phase (Soules, Steiner et al. 1984). LH secretory pattern in women exposed to exogenous progesterone was comparable to LH profiles observed in women in the mid-luteal phase, demonstrating that progesterone plays a central role in regulating the gonadotropin secretory pattern in the luteal phase of normal menstrual cycle (Soules, Steiner et al. 1984). These early findings were consistent with the then recent observations in sheep that estradiol had an inhibitory effect on LH pulse amplitude while LH pulse frequency was decreased by progesterone administration (Goodman and Karsch 1980). These inhibitory effects on gonadotropin secretion was subsequently shown to be mediated through the classic progesterone receptor (PR) although the inhibitory effect of progesterone on LH pulse frequency was rapid and detectable within one pulse (Skinner, Evans et al. 1998). Moreover, the suppressive effect of progesterone on LH pulse frequency was diminished in the context of estrogen deficiency while co-administration of estradiol in this context restored it (Skinner, Evans et al. 1998), suggesting an interplay

between the two sex-steroids. However, the presence of PR expression on only a small subset of GnRH neurons (Fox, Harlan et al. 1990; King, Tai et al. 1995) has led to the notion that intermediary neurons and/or neurotransmitters are involved in the transduction of progesterone inhibitory signals to GnRH neurons. Immunohistochemical studies have lent further support to this notion with only one out of over seven hundred GnRH perikaya co-expressing GnRH and PR, while also demonstrating that PR immunoreactivity is observed in the preoptic area and the arcuate nucleus (Skinner, Caraty et al. 2001), - the latter being a key hypothalamic location involved in GnRH pulse frequency modulation. The importance of the ARC in the modulation of LH pulse frequency has been further characterised recently - the application of micro-implants containing progesterone receptor antagonist (RU486) to ovine hypothalami diminished the inhibitory effect of systemically administered progesterone on LH pulse frequency (Goodman, Holaskova et al. 2011).

Progesterone feedback on GnRH neurons is also mediated by endogenous opioids through the κ opioid receptor (Goodman, Coolen et al. 2004). There is indirect evidence that KNDy neurons in the ARC hypothalamus play a role in mediating progesterone feedback on GnRH through dynorphin, a specific endogenous agonist at the κ receptor (Lehman, Coolen et al. 2010). Progesterone receptors have been demonstrated to be co-localised with dynorphin in the KNDy neuronal population in the ARC (Foradori, Coolen et al. 2002). Progesterone therapy has also been shown to be correlated with an increase in cerebrospinal fluid dynorphin concentration in ovariectomised ewes (Foradori, Goodman et al. 2005). The number of preprodynorphin mRNA expressing cells decreased in ovariectomised ewes and normalised with progesterone therapy to luteal levels (Foradori, Goodman et al. 2005). Unpublished data from the same group also suggest that the application of progesterone antagonist implants to the ovine ARC disrupted the negative feedback effects of progesterone (Lehman, Coolen et al. 2010).

1.3.2 Stress and Glucocorticoids

Stress is widely considered to be a cause of amenorrhea in disease states like depression, malnutrition, eating disorders, excessive exercise and Cushing syndrome (Chrousos, Torpy et al. 1998; Kalantaridou, Makrigiannakis et al. 2004). Experimental evidence from animals models point towards cortisol-mediated

suppression of gonadotropin secretion as the final common pathway that effects gonadotropin suppression in response to various models of stress. There are also data to suggest that upstream factors in the hypothalamus-pituitary-adrenal axis such as Corticotropin Releasing Hormone (CRH) and Arginine Vasopressin (AVP) (Breen and Karsch 2006) play a mediatory role.

1.3.2.1 Effects of glucocorticoids

While it is clear that severe psychological and physical stress manifests as hypothalamic amenorrhea (a heterogeneous diagnosis of exclusion), determining relative contributions of hypothalamic-pituitary-adrenal axis activation and altered metabolic feedback remains to be elucidated in the human context. However, 24-hour cortisol excretion in women with hypothalamic amenorrhea has been demonstrated to be elevated by 17% although cortisol pulsatility and diurnal rhythmicity were preserved (Berga, Mortola et al. 1989). Evening ACTH and cortisol concentrations are known to be higher in highly trained runners (Luger, Deuster et al. 1987) and overtraining leads to elevated urinary free cortisol (Barron, Noakes et al. 1985). Moreover, in women with anorexia nervosa, Corticotropin Releasing Factor (CRF) concentrations are elevated in the CSF (Kaye, Gwirtsman et al. 1987). Administration of modest doses of exogenous glucocorticoids for a fortnight to eugonadal regular cycling women was associated with a decrease in LH pulse frequency without alteration of LH pulse amplitude, suggesting that glucocorticoids have a negative influence on GnRH pulse frequency (Saketos, Sharma et al. 1993). However, it has to be noted that the corticosteroid regime involved the reversal of normal circadian rhythm with 10 mg hydrocortisone administered in the morning (0700-1000 hrs) and 10 mg between 1900 and 2200 (Saketos, Sharma et al. 1993). In sheep models, exogenous cortisol (25 and 100 μg/kg/hr cortisol) has been demonstrated to show suppression of LH amplitude in a dose dependant manner (Debus, Breen et al. 2002).

Indirect inferences on the effects of cortisol on gonadotropin secretion can also be derived from observations in patients with Cushing's syndrome – a clinical condition associated with excessive cortisol secretion. Exogenous GnRH in women with Cushing's disease preferentially stimulates FSH, with LH remaining largely

unchanged (Boccuzzi, Angeli et al. 1975) with similar findings observed in men (Luton, Thieblot et al. 1977). Remission of Cushing's disease in men has also been observed to be associated with resolution of hypogonadotropic hypogonadism in men (Luton, Thieblot et al. 1977).

Studies in animal models have cast doubt on the assumption that glucocorticoid feedback on the reproductive endocrine axis is exerted at a supra-pituitary location. During studies carried out in juvenile rhesus monkey to assess gonadotropin activity at various reproductive stages, it was serendipitously observed that separation of juvenile animals from their mothers caused a sharp decline in gonadotropin secretion in the former (Pohl, deRidder et al. 1995). This suppression of gonadotropin function was associated with increased serum cortisol concentrations (Pohl, deRidder et al. 1995). Moreover, studies in sheep have shown a decrease in LH pulse altitude in response to exogenous cortisol (Breen and Karsch 2004; Breen, Stackpole et al. 2004; Breen, Oakley et al. 2007), suggesting a pituitary locus for this feedback. This notion was lent further support by the observation that LH responses to pulsatile GnRH is diminished by cortisol (Breen, Oakley et al. 2007). However, direct measurement of GnRH in ovine portal blood following 27 hours of cortisol administration demonstrated a 40% decrease in GnRH pulse frequency (Oakley, Breen et al. 2009). Moreover, whilst cortisol alone had no impact on GnRH pulse frequency in ovariectomised ewes, co-administration of estradiol and progesterone led to a 70% decrease in GnRH pulse frequency, suggesting a modulatory role for ovarian steroids in cortisol induced suppression of the reproductive system (Oakley, Breen et al. 2009). Selective blockade of the type 1 and type 2 glucocorticoid receptors have also demonstrated that the suppressive effect of cortisol on LH secretion is mediated through the type 2 glucocorticoid receptor (Breen, Stackpole et al. 2004; Breen, Oakley et al. 2007). In summary, as with estrogen feedback on the hypothalamic-pituitary unit, it is likely that cortisol exerts a negative input both at the pituitary and at the hypothalamic levels.

Decreased *Kiss1* mRNA expression in the ARC has been reported in rats administered glucocorticoids or exposed to stress (hypoglycaemia, restraint and lipopolysacharide induced immunological stress) (Kinsey-Jones, Li et al. 2009).

Preliminary data also suggest that *Kiss1* mRNA expression is reduced with models of stress (food deprivation and restraint) as well as exogenous glucocorticoid administration in male mice (Wang, Lanjuin et al. 2011). This study also reported the presence of glucocorticoid receptor on murine kisspeptin neurons.

1.3.2.2 Hypoglycaemia as a model of stress

Hypothalamic-Pituitary-Gonadal (HPG) axis activity, GnRH pulsatility in particular, is down regulated by stress. Using hypoglycaemia as a stressor, suppression of GnRH pulsatility has been demonstrated in rodents (Cagampang, Cates et al. 1997; Cates and O'Byrne 2000), sheep (Clarke, Horton et al. 1990; Adam and Findlay 1998) and primates (Chen, O'Byrne et al. 1992; Chen, Ordog et al. 1996). Estrogen has been shown to exacerbate this stress-related suppression of GnRH secretion (Cagampang, Cates et al. 1997), and adrenomedullectomy prevents this inhibitory effect (Cagampang, Cates et al. 1997), suggesting sympathetic stimulation/cortisol activity as a causative mechanism.

In human studies, LH and testosterone concentrations in serum have been shown to decrease in response to hypoglycaemic stress irrespective of the insulin dosage infused, suggesting hypoglycaemia rather than circulating insulin levels to be the aetiology of HPG axis suppression (Oltmanns, Fruehwald-Schultes et al. 2001). By inducing hypoglycaemic stress repetitively in the same human subject, it has also been shown that there is no short term adaptation or desensitisation of the HPG axis to stress (Oltmanns, Peters et al. 2005).

1.3.3 Nutrition and metabolism

A link between energy balance and reproductive function enables organisms to survive to reproductive maturity and to withstand the energy needs of parturition, lactation and other parental behaviour (Schneider 2004). This link optimises reproductive success under fluctuating metabolic conditions, ultimately ensuring the survival of the species (Schneider 2004).

1.3.3.1 Acute malnutrition

Acute effects of decreased food intake on LH pulsatility have now been demonstrated in a number of species (Schneider 2004). Periods of fasting as short as 1-2 days significantly decrease LH pulsatility and re-feeding rapidly restores normal LH secretion in rhesus monkeys (Schreihofer, Amico et al. 1993). In women, calorie restriction decreases LH pulse frequency and increases LH pulse amplitude (Loucks, Verdun et al. 1998). Graded calorie restriction (10, 20, 30 or 45 kcal/kg Lean Body Mass per day) in women with regular menstrual cycles have demonstrated a threshold for energy-deficit dependant LH suppression – only daily energy availability less than 30 kcal/kg decreased LH pulse frequency (and increased LH pulse amplitude) (Loucks and Thuma 2003). Moreover, calorie reduction was also associated with an amplification of nocturnal decrease in LH pulse frequency (Loucks and Thuma 2003). Leptin, a satiety hormone, whose levels drop rapidly in response to fasting in human subjects (Boden, Chen et al. 1996; Weigle, Duell et al. 1997) may play a central role in facilitating this inter-regulation. Administration of recombinant leptin increased LH pulse frequency and mean LH in hypothalamic women (Welt, Chan et al. 2004). In healthy men, recombinant leptin prevents fasting-induced drop in testosterone and LH pulsatility (Chan, Heist et al. 2003).

Nutritional deficiency has also been demonstrated to suppress gonadotropin secretion in males of a number of species (Bergendahl and Veldhuis 1995). Three and a half days of fasting by young men effected decreases in LH pulse frequency, pulse mass-per-burst and mean LH, while these parameters were unchanged in older men (Bergendahl, Aloi et al. 1998). Orderliness of LH secretion (approximate entropy) increased with fasting in younger men, but sensitivity to 10 μg GnRH was not affected by fasting status or age (Bergendahl, Aloi et al. 1998). Fasting also led to significant increases in cortisol (Bergendahl, Aloi et al. 1998), making it difficult to attribute the changes in LH to fasting alone. However, augmentation of cortisol in response to stress is well-established and has been demonstrated to be due to increased secretion of cortisol, rather than impairment of its metabolic clearance (Bergendahl, Vance et al. 1996). Moreover, there is a shift in the diurnal pattern of cortisol secretion in models of stress, with peak concentrations observed in the early

afternoon (and not early in the morning as seen in normal subjects) (Bergendahl, Vance et al. 1996).

Food deprivation is associated with a decrease in hypothalamic *Kiss1* mRNA expression in rats, with concomitant increase in *Kiss1R* mRNA (Castellano, Navarro et al. 2005). Time-series studies have shown that decrease in hypothalamic *GnRH* mRNA expression in fasting is preceded by a decrease in *Kiss1R* mRNA expression (Luque, Kineman et al. 2007). Moreover, exogenous kisspeptin restored vaginal opening, a marker of sexual maturation, in malnourished rodents (Castellano, Navarro et al. 2005). Animal models of type 1 diabetes, characterised by insulin deficiency and thus impaired cellular nutrition, demonstrate hypogonadotropic hypogonadism and decreased *Kiss1* mRNA expression (Castellano, Navarro et al. 2006). Repetitive administration of kisspeptin to these rodents with streptazocin-induced diabetes increased prostate and testis weight (Castellano, Navarro et al. 2006). Notably, LH response to kisspeptin in diabetic and control animals were comparable and exogenous kisspeptin normalised the diminished post-orchidectomy rise in LH observed in diabetic male rats (Castellano, Navarro et al. 2006).

1.3.3.2 Chronic malnutrition

Clinical observational studies of growing children carried out in the late 1960s demonstrated that the onset of puberty was triggered by the achievement of a critical body weight and not merely by chronological age – suggesting that pubertal development is triggered by metabolic signals (Frisch and Revelle 1970). Subsequent studies were in keeping with these observations, but additionally identified that 'the degree of fatness' rather than the total body weight alone was critical in the onset of menarche as well as the restoration of menstrual cycles in secondary amenorrhea (Frisch and McArthur 1974). Observations in children with cystic fibrosis (a disease state associated with net calorie deficit in the majority of patients), menarche was found to be correlated with the degree of illness and body fat content (Moshang and Holsclaw 1980). In keeping with these observations, pre-menarche athletic training has been found to be associated with later menarche, in comparison to control population (Frisch, Gotz-Welbergen et al. 1981). These studies led to the emergence of 'critical fat' hypothesis – i.e., that a hypothetical threshold exists for body fat

content exists below which reproductive function is compromised. Hypothalamic signalling pathways relaying nutritional status to the reproductive endocrine axis remained enigmatic until the discovery of leptin, a hormone secreted by adipocytes was shown to be a key metabolic signal modulating reproduction (Barash, Cheung et al. 1996). LH pulse frequency, pulse amplitude and mean serum LH concentrations increases with leptin administration in non-human primates (Finn, Cunningham et al. 1998) and rodents (Nagatani, Guthikonda et al. 1998). However, it has to be noted that this stimulation of gonadotropin is only manifest when the HPG axis has been suppressed by fasting.

However, in situ hybridization studies of hypothalami from multiple species have not demonstrated the presence of leptin receptors on GnRH neurons (Cunningham, Clifton et al. 1999), suggesting the need for an intermediary neuronal network to relay leptin signalling to the GnRH neuron (Quennell, Mulligan et al. 2009). Proopiomelanocortin, neuropeptide Y, and cocaine- and amphetamine-regulated transcript (CART) expressing neurons have been demonstrated to play at least a part in mediating this signalling (Quennell, Mulligan et al. 2009). A role for kisspeptin in this pathway was first suggested following the demonstration of decreased *Kiss1* expression in fasted rodents (Castellano, Navarro et al. 2005) and the normalisation of hypogonadotropic hypogonadism in energy deficient animal models with leptin administration (Castellano, Navarro et al. 2006). These findings have since been replicated in ovine models and kisspeptin neurons were subsequently demonstrated to express leptin receptors (Backholer, Smith et al. 2010). Moreover, kisspeptin neurons communicate with POMC and neuropeptide Y neurons and are able to modulate expression of relevant genes in these cells (Backholer, Smith et al. 2010) – suggesting that kisspeptin's role in the pathway that links reproductive function with metabolic input might be complementary, at least in part, to the roles played by other neuronal systems. Recent studies in leptin deficient rodent models have demonstrated that kisspeptin and GnRH neurons are not activated during induced LH surge (Quennell, Howell et al. 2011) – suggesting that afferent leptin input to the hypothalamic-pituitary gonadal axis is upstream of kisspeptin neurons. Furthermore, this study has also shown that in mice models prone to diet-induced infertility, high

fat diet administration and resultant obesity is associated with down regulation of kisspeptin expression (Quennell, Howell et al. 2011).

Selective deletion of LepR from hypothalamic *Kiss1* neurons in mice was not associated with adverse effects on puberty or fertility (Donato, Cravo et al. 2011). Although methodological issues such as incomplete knock out of hypothalamic KNDy and GnRH neurons, this finding suggests that direct leptin signalling in Kiss1 neurons is not required for these processes. It has to be noted in this context that Estrogen induced Fos expression in GnRH neurons and produced a GnRH-dependent LH surge in GPR54 knock out rodents (Dungan, Gottsch et al. 2007). Taken together, these results suggest that there are pathways other than Kisspeptin-GPR54 system that stimulate the GnRH neuronal population and Leptin's effects are mediated by those alternate pathways. Studies in Ewes have demonstrated that reciprocal connections exist between kisspeptin cells and NPY and POMC cells (Backholer, Smith et al. 2010). Further work is required to elucidate the pathways that integrate leptin signalling with GnRh/LH secretion(Carol F 2012) .

1.3.4 Neurokinin B

A hypothalamic role for neurokinin B in the regulation of reproduction came to be established when genetic studies in patients from consanguineous families with normosmic idiopathic hypogonadotropic hypogonadism were found to have missense mutations in *TAC3* (that encodes neurokinin B) and *TACR3* (that encodes neurokinin B receptor) (Topaloglu, Reimann et al. 2009). *In vitro* characterisation of these receptors demonstrated diminished intracytosolic calcium response to neurokinin B (Topaloglu, Reimann et al. 2009). Men with these mutations had micropenis – suggesting inadequate intrauterine and post-natal testosterone input (Topaloglu, Reimann et al. 2009). A number of cases of NK3 and NK3R receptor mutations, both homozygous and heterozygous, associated with pubertal dysfunction have since been described (Guran, Tolhurst et al. 2009; Gianetti, Tusset et al. 2010; Young, Bouligand et al. 2010). Association of neurokinin B signalling defects with micropenis in males was demonstrated in these studies too, while the largest series amongst these also suggest that many such patients also demonstrate at least partial

reversal of hypogonadism in adult life (Gianetti, Tusset et al. 2010). The small sample size employed in these studies makes it difficult to make meaningful comparisons with other causes of IHH. However, the reported reversal rate in patients with neurokinin B signalling deficiencies, 80% (Gianetti, Tusset et al. 2010) and 15% (Topaloglu and Semple 2011) , are higher than that observed in other forms of IHH (Pitteloud, Acierno et al. 2005; Raivio, Falardeau et al. 2007).

Neurokinins (A&B) are members of the tachykinin family of peptides that also include Substance P (SP) and stimulate three related GPCRs with Gq-coupled signalling (Topaloglu and Semple 2011). Although these receptors NK1R, NK2R, and NK3R (encoded by *TACR1, TACR2* and *TACR3*) are not specific for individual tachykinins, NK3R is considered to show *in vivo* specificity for neurokinin 3 (Sandoval-Guzmán and E. Rance 2004; Topaloglu and Semple 2011).

The association of hypogonadotropic hypogonadism with neurokinin B signalling defect in humans is in contrast to observations made in neurokinin B receptor knock-out rodent models (Kung, Crawley et al. 2004; Nordquist, Delenclos et al. 2008; Topaloglu, Reimann et al. 2009) not demonstrating a GnRH deficient phenotype. However, it has to be noted that Tac2 (the mouse orthologue of human TAC3) knock-out is yet to be studied (Topaloglu and Semple 2011).

Interactions between kisspeptin and neurokinin B systems have also been studied recently in rodents – with divergent findings. Central and peripheral administration of neurokinin B to intact wild-type rodents have shown no effect on circulating luteinising hormone (LH) in a recent study (Corander, Challis et al. 2010) while in earlier studies, the central administration of Senktide, a selective NK3 receptor agonist had demonstrated inhibition (rather than stimulation) of LH secretion in ovariectomised rats (Sahu and Kalra 1992; Sandoval-Guzmán and E. Rance 2004). *Ex vivo* co-stimulation of rat hypothalamic explants with kisspeptin and neurokinin B yielded lower LH stimulation than kisspeptin alone (Corander, Challis et al. 2010). Administration of Senktide evoked potent LH secretion in intact animals with over two-fold increase from baseline in both diestrus and proesterus (Navarro, Castellano et al. 2011). However, ovariectomised rats demonstrated a decrease in LH secretion, whilst the parallel arm of ovariectomised rats treated with a physiological dose of

estrogen showed an increase in LH (Navarro, Castellano et al. 2011). Interestingly, similar differences in responses based on sex steroid status have been demonstrated with other neurotransmitters. Examples include neuropeptide Y, opioids and glucocorticoids – as discussed in detail in section 1.3.5.

A ten-fold increase in c-fos expression in *Kiss1* neurons located in the ARC was also observed following Senktide administration, suggesting that neurokinin B signalling can activate KNDy neurons (Navarro, Castellano et al. 2011). Intracerebroventricular administration of kisspeptin and neurokinin B to rodents were also shown to effect larger increases in LH in comparison to kisspeptin alone (Corander, Challis et al. 2010). Furthermore, the stimulatory effect of senktide, (selective agonist of neurokinin B) was abolished in Gpr54 knock-out male (Garcia-Galiano, van Ingen Schenau et al. 2012), further suggesting that Neurokinin B exerts its action upstream of kisspeptin signalling.

Studies in ruminants have demonstrated the modulatory effect of sex steroids on neurokinin B signalling at the hypothalamus (Billings, Connors et al. 2010). In ovariectomised goats, neurokinin B stimulated LH secretion only after pre-treatment with both estrogen and progesterone (Wakabayashi, Nakada et al. 2010). Arcuate electrical multi-unit activity corresponded to LH secretion, suggesting a hypothalamic site of neurokinin B signalling as well as a potential role for this neuropeptide in GnRH pulse generation (Wakabayashi, Nakada et al. 2010). Unlike studies involving administration of kisspeptin, not all episodes of electrical activity were associated with LH release (Wakabayashi, Nakada et al. 2010). Whilst neurokinin B increased LH pulse amplitude following central administration, no difference in pulse frequency was observed (Billings, Connors et al. 2010). Taken together, these observations suggest that KNDy neurons exert a stimulatory effect on GnRH pulse frequency through neurokinin B (Wakabayashi, Nakada et al. 2010). However, it has to be noted that the observations in rodents are divergent. Intracerebroventricular administration of the selective neurokinin-3 receptor agonist, senktide (100-600 pmol), caused a dose-dependent suppression of LH pulses and multiunit activity volleys in ovariectomized (OVX) and OVX 17β-estradiol-replaced rats (Kinsey-Jones, Grachev et al. 2012).

Studies in primates have further strengthened knowledge of the role played by neurokinin B in the hypothalamic regulation of reproduction. Pre-treatment with GnRH antagonists abolished the stimulatory effect of neurokinin B, demonstrating its site of action to be at or above the GnRH receptor (Ramaswamy, Seminara et al. 2010). Comparative studies of kisspeptin and neurokinin B in gonadectomised rhesus monkeys primed with pulsatile GnRH have demonstrated the amplitude of stimulatory LH response evoked by kisspeptin to be higher (Ramaswamy, Seminara et al. 2010). Pre-treatment with a specific neurokinin B antagonist abrogated neurokinin B's stimulatory effect on LH secretion whilst having no impact on kisspeptinergic stimulation of LH secretion in these primate models (Ramaswamy, Seminara et al. 2010). This notion that kisspeptin exerts its stimulatory effect on LH independent of Neurokinin B stimulation is further supported by preservation of this effect in rhesus monkeys in whom LH stimulatory responses to Senktide were desensitised by repeated administration (Ramaswamy, Seminara et al. 2010; Ramaswamy, Seminara et al. 2011). Although it has been suggested that the order of magnitude of stimulation of LH by kisspeptin and neurokinin B are comparable (Topaloglu and Semple 2011), LH excursion was higher in primates administered boluses of kisspeptin (Ramaswamy, Seminara et al. 2010). Repeated administration of kisspeptin bolus as hourly pulses demonstrated persistence of LH response without tachyphylaxis, unlike neurokinin B (Ramaswamy, Seminara et al. 2010). The latter observation would suggest that desensitisation of LH responsiveness to repeated administration of Senktide occurs at a level above gonadotrope GnRH receptor. This notion is supported by recent studies where kisspeptin stimulation of LH release was preserved in rhesus monkeys desensitised to neurokinin B stimulation by continuous Senktide administration (Ramaswamy, Seminara et al. 2010).

In summary, the effects of NKB on GnRH/LH secretion appear to be dependent on the species studied and the prevailing gonadal steroid mileu. Studies in large mammals (ruminants and primates) suggest a stimulatory role for NKB, whilst the rodent data is not consistent. These emphasise the limitations of using animal models in studying human reproductive function.

1.3.5 Opiates and other neuropeptides

A large number of neuropeptides and other substances have been shown to influence GnRH-gonadotrope function (Urban, Evans et al. 1988; Evans, Sollenberger et al. 1992). Of these, the role played by the hypothalamic opioid peptide system has been investigated most in the human context. When a specific opioid receptor antagonist, naltrexone, was administered to healthy men whose LH pulse frequency had been iatrogenically reduced with DHT infusions, a restoration of LH pulse frequency was observed (Veldhuis, Rogol et al. 1984). Naltrexone also restored LH pulse frequency and LH pulse amplitude in estradiol treated men, suggesting that opiatergic input and sex-steroid milieu regulate GnRH function in a co-ordinated manner (Veldhuis, Rogol et al. 1984). These findings are consistent with earlier studies involving the administration of another opioid antagonist (naltrexone) to women during different phases of normal menstrual cycle – where stimulation of LH secretion was observed in the late follicular and mid-luteal phase, but not in the follicular phase (Quigley and Yen 1980). In women treated with oral medroxyprogesterone acetate (Provera), naloxone therapy has also been demonstrated to restore LH concentrations to pre-Provera levels (Casper and Alapin-Rubillovitz 1985). These observations are also consistent with localisation of human β endorphins to the ARC nucleus in the human brain (Wilkes, Watkins et al. 1980). Perhaps predictably, attempts to control hot flushes in post-menopausal women using naloxone therapy were not successful (DeFazio, Verheugen et al. 1984). These observations are in keeping with studies in oophorectomised women, where no changes in LH concentrations were observed following naloxone administration unless co-administered with an estrogen or progestin (Shoupe, Montz et al. 1985). Preservation of naloxone-mediated increase in LH frequency in gonadectomised individuals with testicular feminisation syndrome also demonstrate that androgen action is not obligatory for opiatergic suppression of GnRH pulse frequency (Veldhuis, Rogol et al. 1985). Taken together, these studies have germinated the notion that endogenous opioids play a central role in the control of the menstrual cycle (Ferin and Vande Wiele 1984) and that opioid input has an inhibitory effect on GnRH –pituitary output.

Interplay between opioid and sex-steroid input demonstrated in the human setting has been investigated in depth in animal models, especially in exploring differential

effects of opioid receptor subtypes (β, μ and δ) and their anatomical distribution. A subset of GnRH neurons in the ovine medial basal hypothalamus demonstrated increased Fos/Fos Related Antigen expression following non-specific opioid antagonist therapy, suggesting preferential suppression of these GnRH neurons by opioids (Boukhliq, Goodman et al. 1999). Administration of receptor specific opioid antagonists to ovariectomised sheep have further demonstrated that μ-receptors, (but not δ- or κ-receptors), play an inhibitory role in the regulation of GnRH surge (Walsh and Clarke 1996). However, κ (but not μ or δ) receptors play a part in the regulation of LH secretion during follicular and luteal phases in ewes, as evidenced in investigations involving the administration of κ – specific ligand, dynorphin (Goodman, Coolen et al. 2004). Furthermore, nearly 90% of all GnRH perikaya at the MBH are reported to be apposed to dynorphin (κ –ligand) containing varicosities in dual immunohistochemical analysis of ovine hypothalami, while electron microscopy has demonstrated dynorphinergic synapses with GnRH perikaya (Goodman, Coolen et al. 2004).

In 2007, it was demonstrated that dynorphin and kisspeptin are co-localised along with Neurokinin B, another neuropeptide modulator of GnRH secretion in the same neuronal population within the ARC nucleus (Goodman, Lehman et al. 2007). Over three quarters of ARC dynorphin neurons co-expressed kisspeptin and vice versa, while most of the kisspeptin neurons also expressed Estrogen Receptor α and Progesterone Receptor (Goodman, Lehman et al. 2007), suggesting that this neuronal population (KNDy neurons - Kisspeptin, Neurokinin-B, Dynorphin neurons) mediates sex-steroid modulation of GnRH secretion (Goodman, Lehman et al. 2007). This population of neurons is also distinct from appetite regulating agouti-related peptide, Neuropeptide Y, melanocortin and β endorphin neurons and shows sexual dimorphism, with ewe hypothalami having more Neurokinin-B neurons in the ARC (Goodman, Lehman et al. 2007) .

1.3.6 Prolactin

The association between hyperprolactinemia and amenorrhea has long been established, accounting for around 14% of secondary amenorrhea in some series with clomiphene non-responsiveness and hypogonadotropism (Bohnet, Dahlen et al. 1976). Hyperprolactinemia has also been reported in a third of women presenting

with infertility (Molitch 1985) and increased prolactin secretion plays a central role in lactational amenorrhea (Diaz, Seronn-Ferre et al. 1989). In men, hyperprolactinemia is evident in 16% of men with erectile dysfunction and 11% of men with oligospermia (De Rosa, Zarrilli et al. 2003). Decreased LH pulse frequency (and by inference GnRH pulse frequency) and increased pulse amplitude that characterises hyperprolactinemia responds to bromocriptine therapy, with increased pulse frequency observed in some patients within few days of therapy (Moult, Rees et al. 1982). Moreover, LH pulses are reported to be more irregular in hyperprolactinemic women and bromocriptine therapy increases regularity of LH pulses (Sauder, Frager et al. 1984). Pulsatile GnRH therapy has also been shown to restore ovulation and normal luteal function in bromocriptine resistant hyperprolactinemic women (Polson, Sagle et al. 1986; Lecomte, Lecomte et al. 1997), suggesting that the central mechanism by which prolactin exerts its hypophysiotropic inhibition is through a reduction of GnRH pulse frequency.

Neuroendocrine pathways by which prolactin inhibits GnRH pulse frequency remain to be fully elucidated. A direct action of prolactin on the GnRH neuronal network is possible as GT1 cells (an immortalised GnRH cell line), express prolactin receptors and GnRH biosynthesis in these cells is inhibited by prolactin (Milenković, D'Angelo et al. 1994). However, whether these findings can be applied to GnRH neurons *in vivo* remains unclear as adult GnRH neurons do not express prolactin receptor (Grattan, Jasoni et al. 2007). Prolactin has been demonstrated to influence other neuronal systems – including GABA (Kolbinger, Beyer et al. 1992), β endorphins (Sarkar and Yen 1985), neuropeptide Y (Li, Chen et al. 1999) and dopaminergic systems (Moore 1987). Whilst all these systems may have a role to play, dopaminergic inhibition has been shown to correlate with menstrual activity and is inhibited by acute sucking and lactation (Moore 1987).

Studies in rodent models suggest that kisspeptin neurons in the ARC nucleus modulate dopamine release from dopaminergic neurons, thereby regulating prolactin secretion (Szawka, Ribeiro et al. 2010). In keeping with this, *Kiss1* expression is decreased in lactation (a physiological state associated with hyperprolactinaemia) (Yamada, Uenoyama et al. 2007). Recent unpublished data suggest that KNDy

neurons in the ovine arcuate nucleus also express prolactin receptors (Li, Clarke et al. 2011).

1.3.7 Acute and chronic illness

Chronic illness is associated with delayed puberty (Pozo and Argente 2002). Acute critical illness is also associated with hypogonadotropic hypogonadotropism, as demonstrated in patients admitted to intensive care facilities (Van den Berghe, Weekers et al. 2001). Although this population is likely to be a heterogeneous cohort with diverse pathology, critically ill patients admitted to intensive care units are in a catabolic state often associated with an acute inflammatory response. Repeated administration of GnRH to critically ill men failed to fully normalise testosterone, suggesting multi-level impairments in the hypothalamic-pituitary gonadal axis (Van den Berghe, Weekers et al. 2001). Recent studies have shown a role for the kisspeptin-GPR54 system in mediating decreased GnRH secretion associated with systemic inflammation (Iwasa, Matsuzaki et al. 2008; Castellano, Bentsen et al. 2010). Decreased *Kiss1* expression has been described in the ARC nucleus of rats in whom gonadotrophic suppression was induced by administration of lipopolyscaharide (LPS), a glycolipid secreted by gram-negative bacteria that triggers inflammation through multiple pathways (Iwasa 2008). Administration of kisspeptin was shown to reverse iatrogenic hypogonadotropism in these animals (Iwasa 2008). It has also been demonstrated that inflammation-mediated suppression of *Kiss1* expression in the ARC nucleus is independent of decreased calorie intake (Castellano, Bentsen et al. 2010).

1.4 Clinical application of GnRH and analogues.

Since the discovery and functional characterization of GnRH, the native peptide as well as analogues have been used extensively for diagnostic and therapeutic use, with annual sales of over two billion US dollars (Millar, Zhu et al. 2000).

1.4.1.1 Diagnostic applications

1.4.1.1.1 Precocious puberty

Provocative testing with GnRH has been widely used in the assessment of pubertal development (Lee 1994). Precocious puberty is defined as the appearance of secondary sexual characteristics in boys younger than nine years and girls younger than eight (Lee 1994; Carel, Eugster et al. 2009). Precocious puberty could arise due to increase in GnRH secretion when it is termed GnRH-dependant (or central precocious puberty) or due to extra-hypothalamic factors such as gonadotropin secreting tumours, adrenal hyperplasia or familial causes when it is termed non-GnRH dependant (Lee 1994). As progressive central precocious puberty is associated with psychological and physical manifestations (Carel, Eugster et al. 2009), an early diagnosis is important. Although baseline and stimulated LH concentrations (following GnRH provocation) overlap prepubertal values, various testing regimes using native GnRH decapeptide or its analogues are used in the assessment with an assay-dependant prepubertal threshold for peak LH defined at 3.3 to 5.0 IU/L (Carel, Eugster et al. 2009). Moreover, provocative tests with GnRH administration have also been proposed as a tool to monitor therapeutic adequacy of gonadotropin suppressive therapy in central precocious puberty (Lee 1994). However, there is a lack of consensus on whether such monitoring adds value over and above clinical observations such as regular monitoring of growth, clinical assessment of pubertal development and radiometric assessment of bone age (Carel, Eugster et al. 2009).

Whilst test regimes involving collection of multiple samples and calculations of LH:FSH ratio have been employed in the past, it has been demonstrated that a single LH value obtained 30 minutes after GnRH administration is sufficient to distinguish central precocious puberty from other causes (Cavallo, Richards et al. 1995).

1.4.1.1.2 Delayed puberty

A lack of pubertal development by two standard deviations above the mean age of pubertal onset in the local population is considered as delayed puberty (Argente 1999). Fourteen and thirteen years of age are widely regarded as the threshold to make the diagnosis in boys (testicular size <4 ml) and in girls (lack of thelarche) respectively (Argente 1999) . The diagnostic challenge in the management of these children is to differentiate pubertal delay that occurs due to inadequate hypothalamic-pituitary activity (hypogonadotropic hypogonadism) and constitutional delay in

puberty (Segal, Mehta et al. 2009). Provocative test with GnRH analogues using 8 IU/L as the cut-off for diagnosis was able to differentiate the two groups in one study (Kauschansky, Dickerman et al. 2002). It has subsequently been shown that combining Human Chorionic Gonadotropin (HCG) stimulation tests with GnRH provocative testing can improve this differentiation (Segal, Mehta et al. 2009).

1.4.1.1.3 Other diagnostic applications

Provocative testing with GnRH has been proposed as a tool to differentiate hypothalamic and pituitary causes of central hypogonadotropism (Swerdloff, Peterson et al. 1978). However, clinical studies have since shown that due to considerable variability in gonadotropin response in pathological settings with marked overlap with responses observed in healthy subjects, GnRH provocative testing may not be useful in the diagnosis of hypothalamic-pituitary-gonadal dysfunction (Mortimer, Besser et al. 1973; Westwood, Butler et al. 2000; Chammas, Chambers et al. 2008). With the development of sensitive and specific assays for gonadotropins, baseline LH and FSH are widely used in clinical practice, despite the theoretical concern that the timing of the sample relative to the underlying pulsatility will influence the observed results.

1.4.1.2 Therapeutic applications

1.4.1.2.1 Pubertal dysfunction

GnRH analogue therapy in precocious puberty is aimed to suppress endogenous GnRH activity and exploits the desensitisation of the hypothalamic-pituitary unit to prolonged GnRH stimulation initially demonstrated in primate models (Belchetz, Plant et al. 1978). Children with peripheral and central precocious puberty were administered GnRH analogues as early as 1979, rapidly translating the findings in animal models (Pescovitz, Comite et al. 1986). Children with central puberty responded to the therapy, while those with peripheral causes did not (Pescovitz, Comite et al. 1986). Moreover, GnRH analogue therapy has been demonstrated to be safe, with pubertal development recommencing once the treatment was withdrawn (Pescovitz, Comite et al. 1986; Manasco, Pescovitz et al. 1988).

Pulsatile GnRH therapy has been demonstrated to initiate pubertal development in boys and girls presenting with delayed pubertal development (Hoffman and Crowley 1982; Stanhope, Pringle et al. 1987). Whilst the requirement for hormonal replacement therapy in these individuals has been previously thought to be lifelong, there is emerging evidence that hypogonadotropic hypogonadism in some of these subjects could be reversed (Quinton, Cheow et al. 1999; Raivio, Falardeau et al. 2007; Ribeiro, Vieira et al. 2007). Prospective studies have shown a 10% reversal rate (95% Confidence Interval 2 -18%) (Raivio, Falardeau et al. 2007) and in post-reversal follow-up (range of duration of follow-up 0.6 to 23.7 years) thirteen of fifteen patients had adult serum testosterone throughout the follow-up period (Raivio, Falardeau et al. 2007). The mechanisms underpinning this apparent plasticity of GnRH neurons remain to be elucidated. However, as non-responders were identified as early as 7±4 weeks following discontinuation of therapy, brief withdrawal of therapy may be warranted in patients with delayed puberty who have achieved adult testosterone concentrations (Raivio, Falardeau et al. 2007). It has to be noted that non-pulsatile treatment with GnRH does not lead to progression of puberty, as observed in a study involving the administration of 500 μg of GnRH twice daily (Brook and Dombey 1979).

1.4.1.2.2 Induction of ovulation

Pulsatile administration of GnRH at physiologic doses and frequency has been demonstrated to induce ovulation in women with anovulatory infertility (Crowley and Mcarthur 1980; Leyendecker, Struve et al. 1980; Skarin, Nillius et al. 1983), providing conception and live birth rates comparable to normal women (Balen, Braat et al. 1994). In women with anovulation associated with polycystic ovary syndrome (PCOS), pulsatile GnRH administration immediately after GnRH-A suppression appears to be more effective (Filicori, Campaniello et al. 1988). While an ovulation rate of 76% and pregnancy rate of 28% per cycle were reported , subsequent studies have shown lower rate of success – at 47% ovulation rate and 10% pregnancy rate (Timmerman-van Kessel, Cikot et al. 2000). When pulsatile GnRH therapy following sex-steroid (combined oral contraceptive pill) suppression of gonadotropin secretion GnRH antagonist therapy was compared with identical pulsing regime preceded by

GnRH analogue therapy, the latter was found to be more effective (Gerhard, Matthes et al. 1993). However, the need for routine use of GnRH pumps in induction of ovulation has decreased with the widespread use of low-dose FSH protocol (Li, Van Esch et al. 2010). These protocols show cumulative live birth rates of up to 74.2%, with multiple live birth rate as low as 2.5% and are also less resource intensive (Li, Van Esch et al. 2010).

In women with polycystic ovarian syndrome, higher serum LH concentrations were observed during the follicular phase in women administered pulsatile LH after GnRH analogue therapy when compared to women administered clomiphene citrate (a selective estrogen receptor modulator –SERM) (Timmerman-van Kessel, Cikot et al. 2000). Higher LH concentrations in the follicular phase are considered to contribute to decreased conception rate and increased early loss of pregnancy (Homburg, Armar et al. 1988). Therapeutic approaches with a more nuanced stimulatory effect on LH may therefore be more beneficial in this context. In the treatment of women with clomiphene resistant anovulatory infertility associated with polycystic ovary syndrome, subcutaneous pulsatile GnRH (administered in a step-wise fashion as GnRH alone, combination of clomiphene citrate and GnRH, and combination of GnRH with gonadotropins) effected high cumulative conception rate with a low rate of multiple pregnancy and ovarian hyper stimulation syndrome (Tan, Farhi et al. 1996).

1.4.1.2.3 Gonadotropin suppression

The ability of continuous GnRH stimulation to suppress gonadotropin release and thereby ovulation has been applied therapeutically in the treatment of endometriosis but requires sex steroid 'add-back' (replacement) to prevent deleterious effects on the skeleton, hot flushes and vaginal dryness (Guzick, Huang et al. 2011). GnRH analogue therapy has also been demonstrated to be useful in reducing uterine fibroid dimensions before surgery (Lumsden, West et al. 1987). Moreover, androgen deprivation therapy using GnRH analogue therapy is the mainstay of the modern management of prostate cancer (Sharifi, Gulley et al. 2010). A number of other clinical applications of GnRH analogues have been reported – including in the

treatment of breast cancer (Mastro, Levaggi et al. 2011), premenstrual syndrome (Di Carlo, Palomba et al. 2001) and microprolactinoma (Rubio, Cabranes et al. 1989).

1.5 Summary

In summary, the hypothalamic GnRH neuronal network integrates developmental, environmental, endocrine and metabolic inputs, thereby modulating the reproductive endocrine axis (Please see figure 1.1). The discovery of GnRH has considerably deepened the understanding of reproductive neuroendocrine physiology, with resultant diagnostic and therapeutic applications. The discovery of kisspeptins and the KNDy neuronal network in the last decade has provided novel insights into the regulation of GnRH secretion and administration of exogenous kisspeptin appears to be a useful tool in probing GnRH neuronal function *in vivo*. Translational clinical studies, exploring kisspeptin responses in various physiological and pathological states are pivotal to exploring and elucidating potential clinical application for kisspeptins.

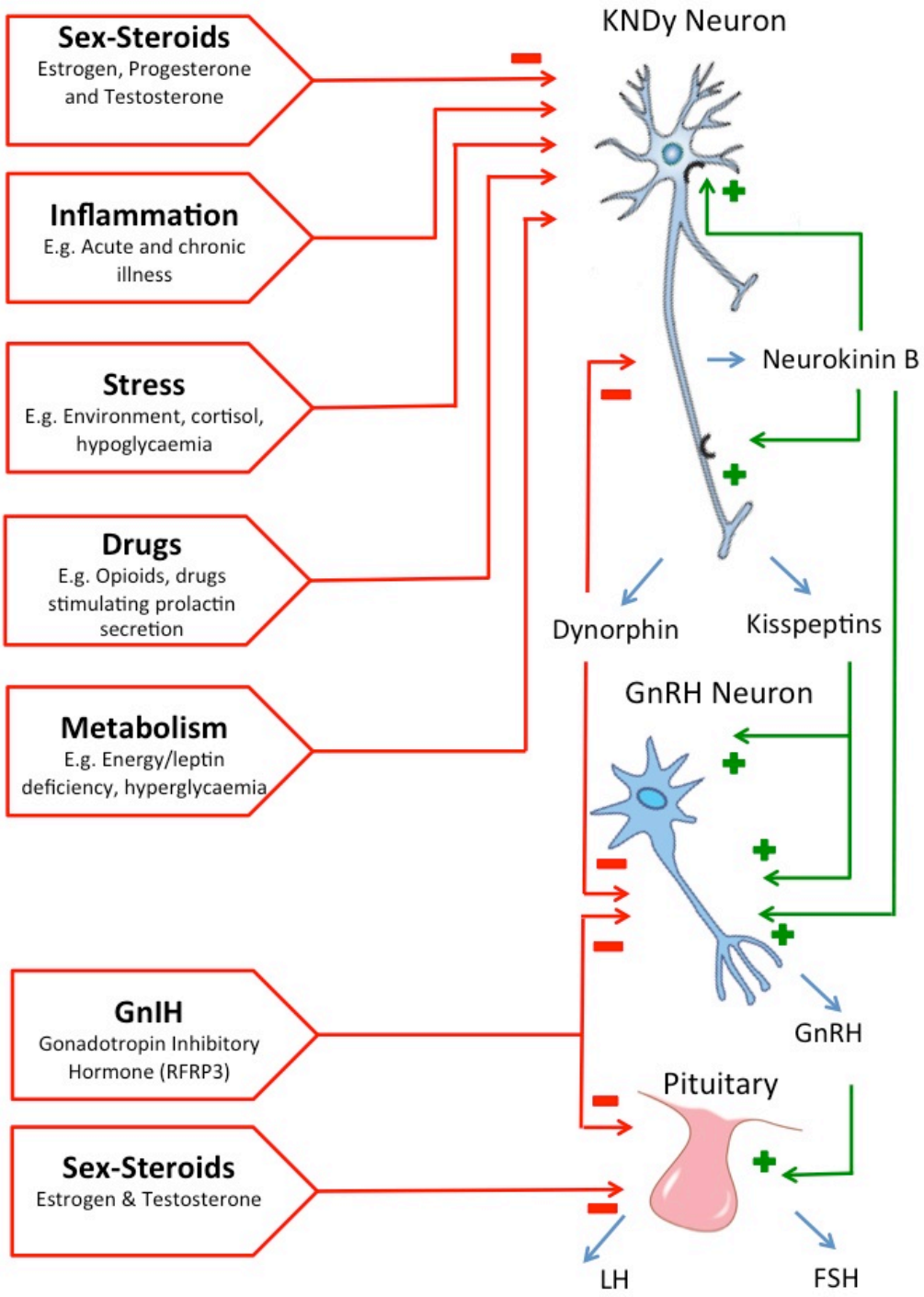


Figure 1.1 Schematic representation of neuroendocrine regulation of gonadotropin secretion.

1.6 Hypotheses

The overarching hypotheses for the work described in this thesis are

1) That exogenous kisspeptin-10, administered as acute boluses, would stimulate LH secretion in men and women. As the sex steroid feedback inhibition of the HPG axis appears to be exerted, at least in part, at the level of the kisspeptin neuron, it was hypothesised that this response to kisspeptin-10 would be preserved in women with a range of prevailing steroid milieu.
2) That continuous infusion of kisspeptin-10 would enhance LH pulse frequency.

2 Materials and Methods

2.1 Ethical Standards and Research Governance

2.1.1 Approvals by ethics committee

All the clinical studies in this thesis are conducted in accordance with the principles of the International Conference on Harmonisation Tripartite Guideline for Good Clinical Practice (ICH GCP) and the Declaration of Helsinki. Studies were approved by a local research ethics committee (Lothian REC Ref 09/S1101/23 and 09/S1101/67).

2.1.2 Regulatory compliance in the *first-in-human* context

Prior to the commencement of these studies, clarification was sought from the Medicines and Healthcare Products Regulatory Agency (MHRA) who confirmed that these studies are not clinical trials of investigational medicinal product (CTIMP). Regulatory approval has hence been sought as physiological studies of a naturally occurring peptide. The University of Edinburgh and NHS Lothian jointly sponsored the studies reported herein.

2.1.3 Informed Consent

Written informed consent was obtained from all volunteers. All potential participants were provided written and oral information and were provided the opportunity to clarify any points they do not understand and, if necessary, ask for more information. Where possible, information leaflets were provided by email or post ahead of the potential participant's initial screening visit to ensure adequate time is available to consider the proposition. Information sheets also carried contact details for an independent endocrinologist.

2.1.4 Confidentiality and data protection

All laboratory specimens, evaluation forms, reports, and other study records were link-anonymised to maintain participant confidentiality. Hard copies are held in an access-controlled area within the Royal Infirmary of Edinburgh while electronic data is held securely on University of Edinburgh servers.

2.2 Recruitment of volunteers

2.2.1 Healthy volunteers

Printed flyers and email invitations were sent to large employers in Edinburgh. Printed flyers were also placed in the New Royal Infirmary of Edinburgh and the Western General Hospital for the attention of visitors and carers. Once a potential recruit expressed an interest in taking part in a study was expressed, further information about the study was provided over telephone or as an email or printed flyer depending on individual preference. Once the prospective volunteer was satisfied with the information provided, they were invited to attend for a screening visit.

2.2.2 Recruitment of volunteers with type 2 diabetes

Participants with diabetes were identified from the diabetes clinics at the Royal Infirmary of Edinburgh and the Western General Hospital. The study was introduced to patients at routine clinic visits by clinical staff involved in their normal care. Subsequently contact was made with those interested and to make arrangements for a fuller discussion of the study and a screening visit.

2.2.3 Recruitment of volunteers with Kallmann Syndrome

Participants with Kallmann Syndrome were identified with the help of the Kallmann Society and Dr. Richard Quinton, Consultant Endocrinologist at Newcastle who kindly provided participants with information about the study. Volunteers were thus drawn across the UK and all studies were carried out in Edinburgh.

2.2.4 Recruitment of patients with Neurokinin B signaling defects

Four patients with TAC3 (NKB) or TACR3 (NK3R) biallelic mutations presenting with delayed puberty were identified by Dr. Jacques Young and enrolled in this study. Subjects were admitted to the Endocrinology and Reproductive Diseases Department (Bicêtre hospital, France) where blood sampling and administration of kisspeptin-10 was carried out.

2.2.5 Screening for eligibility

All subjects who attend for a screening visit had their weight, height and baseline blood tests (including renal function, liver function, full blood count and testosterone/estradiol) obtained. Structured clinical history was obtained to identify pre-existing hypothalamic or pituitary disease.

2.3 Drugs and devices used in the study

2.3.1 Kisspeptin-10

Kisspeptin-10 was custom-synthesised under GMP standards (Bachem GmBH, Weil am Rhein, Germany) and supplied in single use vials of 1 mg each. Purity was assessed by HPLC at 97% with a mass balance of 98.8%. For each participant, kisspeptin-10 was made up within the hour prior to injection by diluting 1 mg of lyophilised kisspeptin-10 in 5ml sterile normal saline. Stability of kisspeptin-10 in solution was assessed by *in-vitro* receptor binding studies comparing pre-incubated and freshly constituted 0.2mg/ml solution (kindly performed by Dr. Antonia Roseweir).

2.3.2 GnRH

Commercially available GnRH (Relefact, Sanofi Aventis, Frankfurt, Germany), supplied in 100 mg single-use ampoules were used for GnRH stimulation tests.

2.3.3 Vehicle

Sterile 0.9% saline was used as vehicle in these studies. Supplies were provided by NHS Lothian Clinical Research Facilities as sourced from various manufacturers.

2.3.4 Intravenous infusion devices

Dedicated infusion devices (Crono PCA, Cane, Rivoli, Italy), designed to deliver low infusion rates were used for kisspeptin-10 administration. To minimise adherence to plastic, syringes and tubing used to administer kisspeptin-10 were coated with the peptide prior to being loaded.

2.3.5 Collection of samples

All samples were drawn through an indwelling intravenous cannula inserted under aseptic precautions. After each sample was drawn, 5 ml of normal saline was injected

into the cannula to prevent coagulation of blood within the sampling system. Before each draw, 2 ml of blood was discarded to avoid contamination by residual saline in the cannula and attached tubing. Samples for hormone assays (LH, FSH, E and T) were collected in vacuum serum tubes (Monovette, Sarstedt UK Ltd, Leicestershire) and batch-processed in the Clinical Research Facility at regular intervals with centrifugation of whole blood 4 degrees C for 10 min at 3000rpm. Pre-labelled aliquot tubes were filled with serum and samples frozen at -20 degree celcius till analysis.

2.3.6 Analysis of samples

Hormone assays were carried out by assay lab technicians attached to the MRC Human Reproductive Sciences Unit using in-house assays. LH and FSH concentrations were determined by ELISA, and testosterone by RIA as previously described (Brady, Anderson et al. 2003). Inter-assay coefficient of variation for all hormonal assays was <5 % at the concentrations measured. Intra-assay coefficient of variation of LH was 2.9%. All samples from each of the study visits were analysed together in duplicate.

2.3.7 Safety and screening bloods

Full blood count, serum electrolytes, renal function tests and liver function tests were carried out at each of the study visits. These samples were sent to the hospital laboratories of NHS Lothian and processed on standard assay platforms.

2.3.8 Statistical software

Data are presented as mean±sem. A two-sided $P < 0.05$ was regarded as statistically significant for all analyses. All primary data are stored as Microsoft Excel files (Microsoft, Redmont, USA). The statistical software package Minitab 16 (Minitab Ltd, Coventry, UK) was used for analysis. Graphs and charts were produced using Graphpad Prism (Graphpad Software, CA, USA). Additional expert input was kindly provided by Ronnie Grant (Graphics Lab, MRC Centre for Reproductive Health) in preparing graphs and figure.

3 Kisspeptin-10 dose-dependently stimulates gonadotropin release in healthy male volunteers

3.1 Introduction

Since their discovery as obligatory elements to normal pubertal development, kisspeptins have been demonstrated to be modulators of GnRH function (section 1.1).

Administration of exogenous kisspeptin stimulates LH secretion in both men and women (Dhillo, Chaudhri et al. 2005; Dhillo, Chaudhri et al. 2007; Jayasena, Nijher et al. 2009). Human studies of kisspeptin have hitherto used the 54-amino acid peptide, kisspeptin-54 (Dhillo, Chaudhri et al. 2005; Dhillo, Chaudhri et al. 2007; Jayasena, Nijher et al. 2009; Jayasena, Nijher et al. 2010). Kisspeptin-54 is cleaved from the 145 amino acid precursor polypeptide encoded by *KISS1* and is further processed to 14, 13 and 10 amino acid (kisspeptin-10) sequences, all sharing the same C-terminal decapeptide RF-amide sequence (Kotani, Detheux et al. 2001). Whilst kisspeptin-10 has intrinsic bioactivity similar to the longer kisspeptin fragments (Kotani, Detheux et al. 2001), it is also characterised by a shorter half-life and more rapid onset of action after iv administration in rodents (Mikkelsen, Bentsen et al. 2009). Kisspeptin-10 also has greater potential for pharmaceutical development as both agonists (Curtis, Cooke et al. 2009) and antagonists (Roseweir, Kauffman et al. 2009; Millar, Roseweir et al. 2010) have been developed based on its decapeptide sequence. However, there have been no studies on the activity of kisspeptin-10 in humans.

3.2 Objective

The present study involved first-in-human studies of kisspeptin-10. The objective was to establish the dose dependency and time course of stimulation of LH and FSH secretion following intravenous bolus doses of kisspeptin-10.

3.3 Methods

3.3.1 Participants

Six healthy male volunteers took part in this acute kisspeptin stimulation study. All volunteers provided informed written consent and the study was approved by a local research ethics committee (Lothian REC Ref 09/S1101/23). The age of healthy male participants was 35.6±3.4 (mean±SEM) and body mass index was 26.1±0.8 kg/m^2, all of whom had a minimum total testicular volume of 40 ml, normal physical examination and normal secondary sexual characteristics. Baseline full blood count, renal function, liver function and electrolytes were within normal limits.

3.3.1 Kisspeptin-10

Kisspeptin-10 was custom synthesised to GMP standards and prepared for administration as described in chapter 2.

3.3.2 Protocol

This study was designed to assess the magnitude and dose-dependency of gonadotropin secretion in response to six intravenous bolus doses (0.01, 0.03, 0.1, 0.3, 1.0 and 3.0 μg/kg) of kisspeptin-10 or vehicle (normal saline). These doses were selected on the basis of a previous study of kisspeptin-54 in healthy male volunteers (Dhillo, Chaudhri et al. 2005). Additionally, based on studies in rodents demonstrating comparable efficacy, an assumption was made that kisspeptin-10 would elicit gonadotropin responses observed with comparable doses of kisspeptin-54. Moreover, as the study involved first-in-human administration of kisspeptin-10 with concentrations reaching supraphysiological levels (albeit only for a short duration based on studies in animal models), the top dose was set at 3.0 μg/kg, ten times the equimolar equivalent of the maximally effective dose of kisspeptin-54 (Dhillo, Chaudhri et al. 2005) in view of potential off-target effects.

All volunteers attended the study in a fasting state and all visits commenced between 0800 and 0900 to avoid diurnal bias. Blood samples were obtained through indwelling intravenous cannulae before (60, 45, 30, 20, 10 min and immediately) and after (10, 20, 30, 45, 60, 75 and 90 min) kisspeptin-10 or vehicle administration.

Doses were administered in increasing order for safety reasons and visits were at least 1 week apart. All volunteers received at least 4 doses and 4 volunteers received all doses. Participants were blinded to the dose of kisspeptin-10.

Blood pressure, pulse and peripheral oxygen saturations were measured every 3 min with standard automated techniques. Full blood count and serum electrolytes, liver function and renal function were checked at each visit.

3.3.3 Hormone assays

Blood samples were centrifuged immediately at 4^{o}C for 10 min at 3000 rpm and serum frozen at -20^{o}C until analysis. LH and FSH were determined by ELISA, and testosterone by RIA as previously described (22). Inter-assay coefficient of variation for all hormonal assays was <5 % at the concentrations measured. Intra-assay coefficient of variation of LH was 2.9%. All samples from each of the study visits were analysed together in duplicate.

3.3.4 Statistical Analysis

For the dose-finding study, 60-min mean LH and FSH concentrations and Area-Under-Curve (AUC, by trapezoid integration) pre and post kisspeptin-10 bolus were calculated. Variance in mean LH and ΔAUC values in the dose-response study was analysed by ANOVA followed by Tukey's simultaneous test. Student's t test was used to analyse changes in mean LH, FSH and testosterone concentrations in the infusion studies. Data are presented as mean±sem. A two-sided $P < 0.05$ was regarded as statistically significant for all analyses. The statistical software package Minitab 16 (Minitab Ltd, Coventry, UK) was used.

3.4 Results

Acute kisspeptin administration elicited LH and FSH responses in a dose-dependent manner.

3.4.1.1 Effect of Kisspeptin-10 on LH secretion in healthy male volunteers – time course

Intravenous injections of kisspeptin-10 elicited a rapid increase in LH in all volunteers, with peak concentrations seen by 45 min post injection for all doses studied. The maximum LH stimulation was seen following the 1µg/kg bolus, achieving peak LH concentration (12.4±1.7 IU/L) by 30 min (figures 3.1).

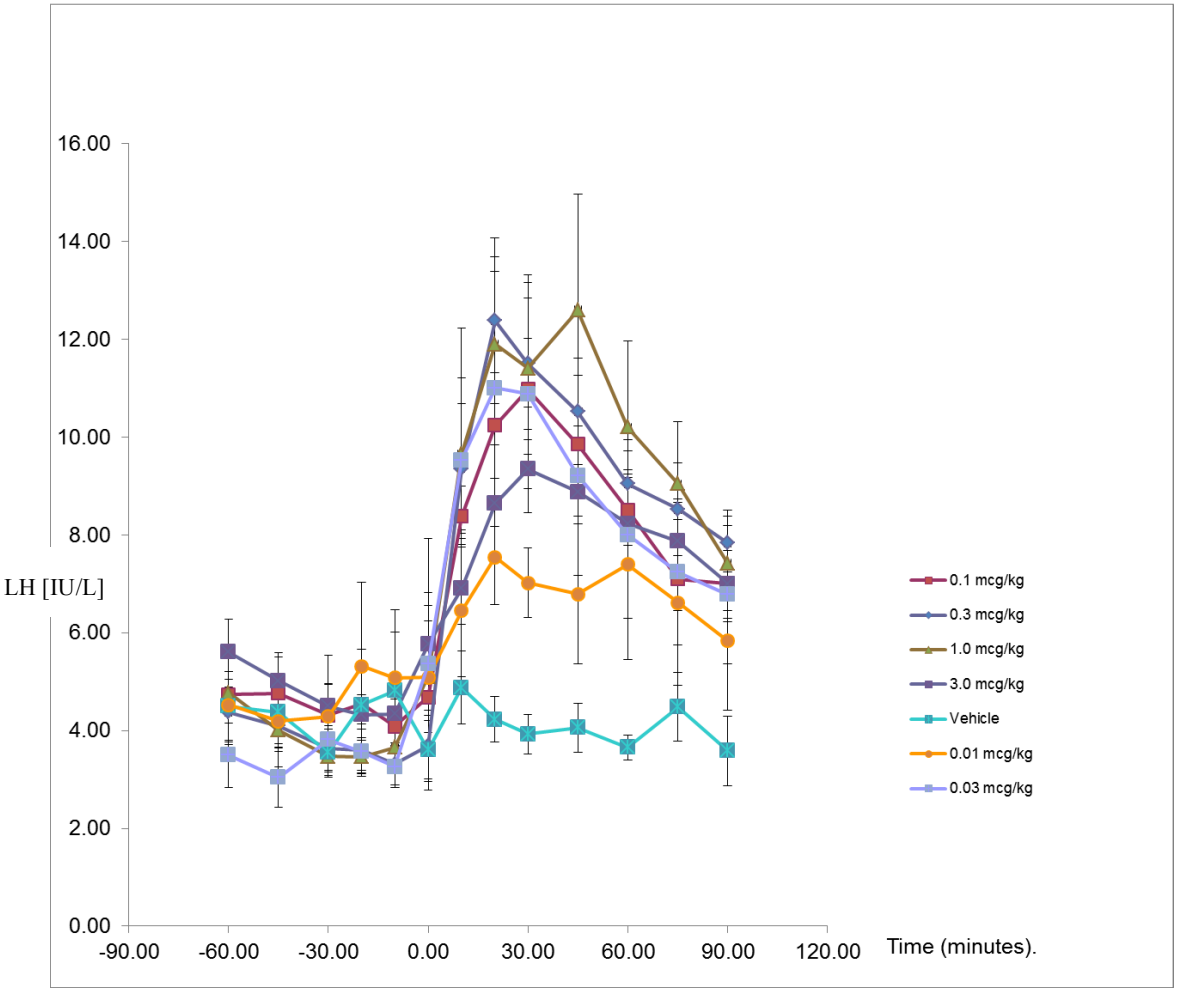


Figure 3.1: Effect of Kisspeptin-10 on LH secretion in healthy male volunteers -time course of LH response following all doses of kisspeptin-10 administered as intravenous bolus doses. Kisspeptin-10 or vehicle administered intravenously to healthy volunteers at time 0.

3.4.1.2 Effect of Kisspeptin-10 on LH secretion in healthy male volunteers: LH dose responsiveness

There was a clear dose-dependent increase in LH concentrations in response to kisspeptin-10 ($P<0.0001$). Statistically significant increases were observed following the 0.03, 0.1, 0.3 and 1µg/kg bolus doses ($P<0.01$). There was however no significant increase in LH concentration following the highest dose administered (3.0µg/kg) and mean LH following this dose was significantly less than following 0.3 and 1µg/kg doses (both $P<0.05$). Figure 3.2 summarises baseline LH and peak LH observed following kisspeptin-10 administration for each of the doses studied.

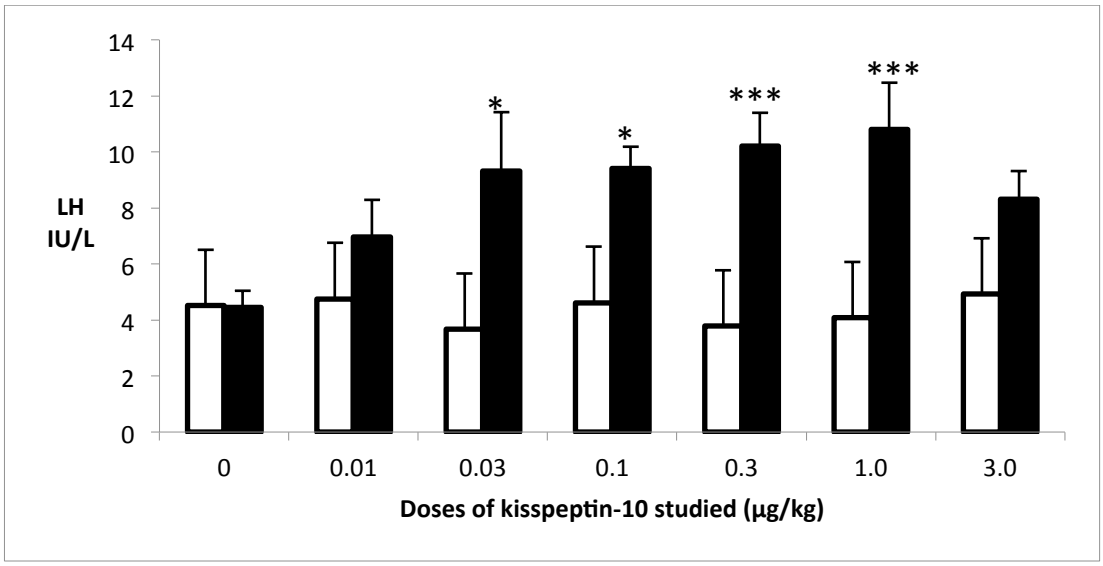


Figure 3.2: Effect of Kisspeptin-10 on LH secretion in healthy male volunteers-analysis of variation of dose-dependent stimulation of LH secretion.

60 min -AUC following kisspeptin-10 administration also showed dose dependence, with 0.1, 0.3 and 1µg/kg doses achieving significant changes compared to vehicle. (Figure 3.3)

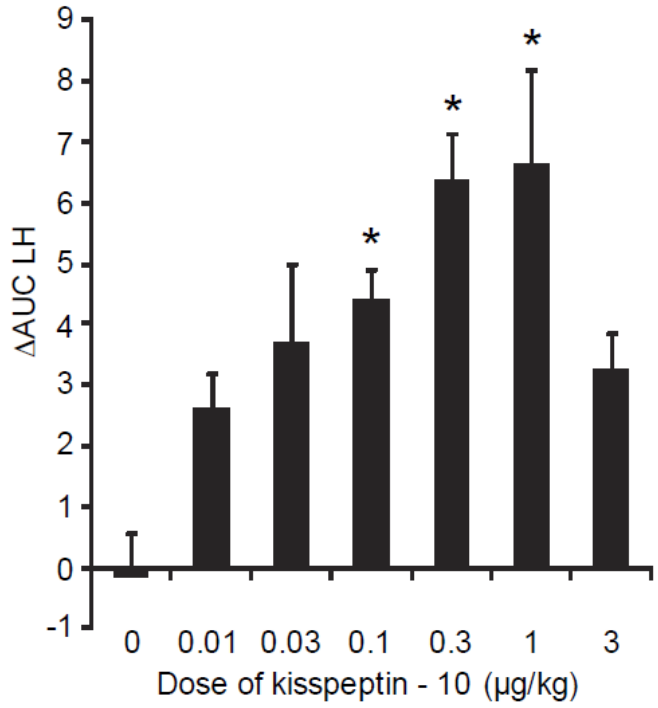


Figure 3.3: Effect of Kisspeptin-10 on LH secretion in healthy male volunteers- analysis of variance of 60-min ΔAUC of LH following kisspeptin-10 administration. Asterisks denote doses significantly different from vehicle (P <0.05). ΔAUC of LH of each dose is compared in the figure to that observed following the administration of vehicle.

3.4.1.3 Effect of Kisspeptin-10 on FSH secretion in healthy male volunteers:

FSH secretion also increased dose-dependently following kisspeptin-10 administration (P = 0.012). The largest increase was observed with 1µg/kg where the mean 60-min FSH rose from 4.6±1.0 to 5.3±1.0 IU/L (Figure 3.4).

Increases were seen in AUC of FSH following 0.3 and 1µg/kg kisspeptin-10, while 3µg/kg again did not result in a significant increase in FSH concentration. The time course of FSH response to kisspeptin-10 stimulation is summarised is illustrated in Figure 3.5.

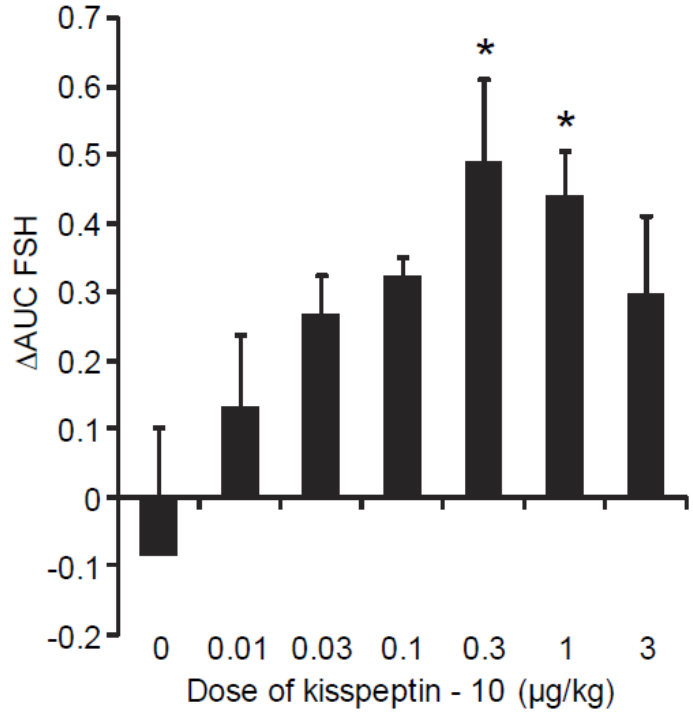


Figure 3.4: Effect of Kisspeptin-10 on FSH secretion in healthy male volunteers- Analysis of variance of 60-min ΔAUC of FSH following administration. Asterisks denote doses significantly different from vehicle (P <0.05).

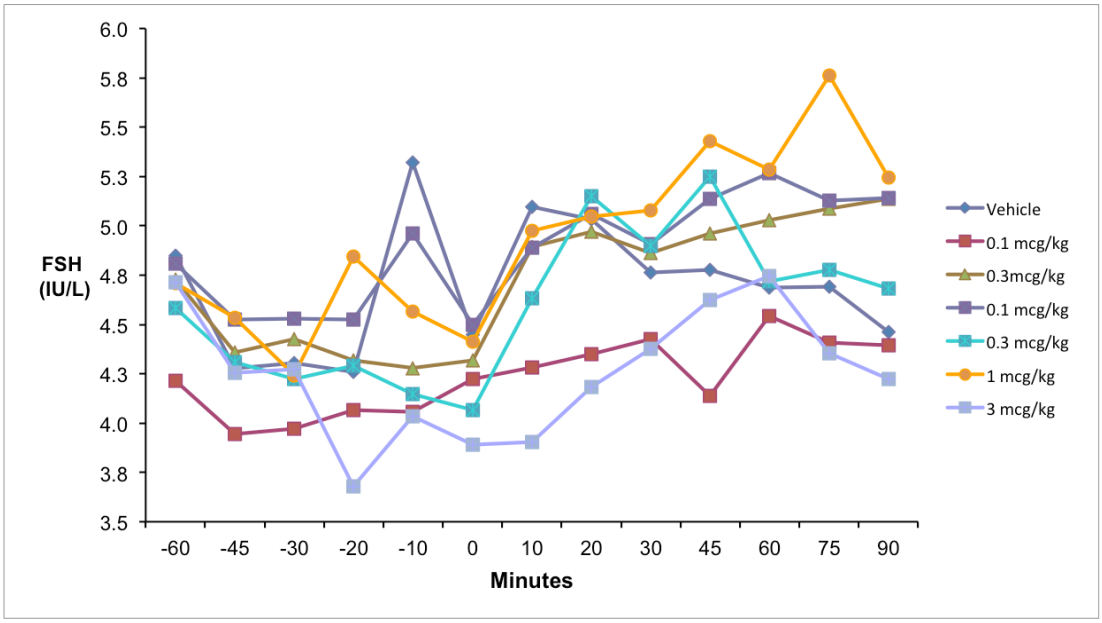


Figure 3.5: Effect of Kisspeptin-10 on FSH secretion in healthy male volunteers- time course of mean LH responses to doses studied.

3.4.1.4 Effect of Kisspeptin-10 on Testosterone secretion in healthy male volunteers

Serum testosterone concentrations showed no statistically significant change with any of the doses studied using this protocol. Figure 3.6 summarises testosterone responses to kisspeptin-10.

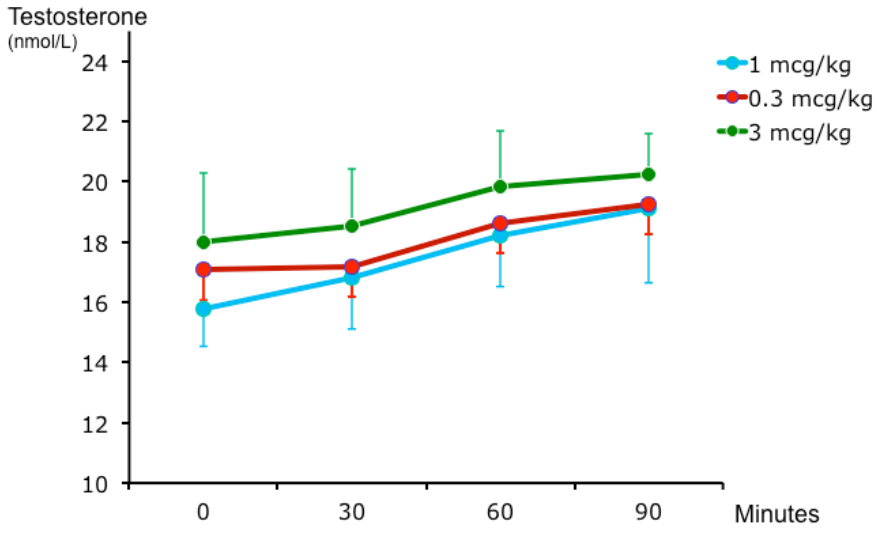


Figure 3.6: Effect of Kisspeptin-10 on LH secretion in healthy male volunteers: Testosterone responses to kisspeptin-10. No statistically significant changes were observed.

3.4.1.5 Effect of Kisspeptin-10 on other pituitary hormones.

Concentrations of Thyroid Stimulating Hormone, Prolactin and Cortisol (used as a surrogate for ACTH) were unchanged following 3 µg/kg kisspeptin-10 administration. These hormones were analysed only following the largest dose studied as any such effects, based on observations in animal models were hypothesised to be GPR-54 mediated, not GnRH dependant effects. Insulin and glucose, measured in view of studies in animal models suggesting kisspeptinergic

potentiation of insulin secretion, were also unchanged (Bowe, King et al. 2009). Growth hormone showed a tendency to decrease but this was statistically non-significant. A post-hoc analysis using paired t-test of baseline (0 min) excluding the subject with suppressed GH concentration at baseline yields statistical significance, but considering the high variability of GH, longer time-series studies in larger number of subjects are needed to clarify this. Table 3.1 and 3.2 details the concentration of these hormones in the subjects studied.

Table 3.1. Glucose and insulin response to an intravenous bolus of 3-µg/kg kisspeptin-10.

Glucose	Time points (minutes)			
Subject No	-30	0	30	60
1	5.4	5.2	5.4	5.4
2	5.1	5.1	5.2	5
3	5.7	5.6	5.5	5.6
4	5.3	5.1	5.4	5.2
5	5.4	5.2	5.1	5.2
6	5.3	5.5	5.1	5.1
Insulin	Time points (minutes)			
Subject No	-30	0	30	60
1	5.4	5	3.8	4.7
2	16.9	10.2	14.8	10.3
3	7.2	6.5	6.1	6.6
4	4.5	4.6	3.9	3.7
5	10.3	11.4	9.2	11.6
6	8.2	7.4	7.4	7.1

Table 3.2. GH, TSH, Prolactin and Cortisol responses to 3 µg/kg intravenous bolus of kisspeptin-10

GH	Time points (minutes)			
S. No	-30	0	30	60
1	0.11	0.12	0.13	<0.10
2	<0.10	<0.10	<0.10	<0.10
3	0.18	0.15	0.11	0.2
4	0.63	0.2	0.13	0.15
5	0.37	0.4	0.1	<0.10
6	0.85	0.35	0.13	<0.10
TSH	Time points (minutes)			
Subj No	-30	0	30	60
1	1.45	1.46	1.32	1.46
2	0.83	0.76	0.70	0.65
3	1.57	1.48	1.44	1.45
4	1.00	0.94	0.85	0.91
5	1.36	1.33	1.27	1.25
6	2.47	2.27	2.27	2.26
Prolactin	Time points (minutes)			
Subj No	-30	0	30	60
1	151	139	142	123
2	79	73	70	73
3	187	195	200	191
4	133	126	112	97
5	88	72	64	60
6	224	203	208	184
Cortisol	Time points (minutes)			
Subj No	-30	0	30	60
1	275	237	230	280
2	279	219	185	219
3	189	210	180	246
4	164	127	111	105
5	221	177	134	126
6	313	264	198	194

3.4.1.6 Adverse effects

Blood pressure, heart rate, peripheral oxygenation, liver function, renal function, haemoglobin, mean corpuscular volume and electrolytes remained stable in all subjects throughout the study period. No adverse events were reported.

3.5 Discussion

We have demonstrated that intravenous administration of kisspeptin-10 boluses result in potent and dose-dependent stimulation of LH secretion in healthy men. Doses of kisspeptin-10 as low as 0.03 μg/kg (23 pmol/kg) elicited a significant rise in LH when compared to vehicle, demonstrating the high potency of kisspeptin-10. This is in keeping with data from experimental animals where intracerebroventricular doses of kisspeptin as small as 1 fmol effected robust LH stimulation (Gottsch, Cunningham et al. 2004). Central rather than pituitary mediation of the effect of kisspeptin on LH secretion has been demonstrated by abolition of the effect of kisspeptin by pre-treatment with a GnRH antagonist (Gottsch, Cunningham et al. 2004; Irwig, Fraley et al. 2004; Plant, Ramaswamy et al. 2006).

The 2.5 fold increase in serum LH from baseline observed following kisspeptin bolus administration in our study is comparable to that observed in men administered equimolar doses of kisspeptin-54 as short infusions (Dhillo, Chaudhri et al. 2005). Moreover, as in human studies using kisspeptin-54 (Dhillo, Chaudhri et al. 2005), the stimulatory effect of kisspeptin-10 on LH secretion in our study was also markedly more pronounced than that on FSH secretion. Early animal studies suggested stimulatory effects on LH and testosterone secretion were seen only with the longer kisspeptin peptide, kisspeptin-54, and not shorter forms (Thompson, Murphy et al. 2006). However, *in vivo* rodent data have subsequently shown equal potency of kisspeptin-10 and kisspeptin-54 (Gottsch, Cunningham et al. 2004; Tovar, Vazquez et al. 2006) and in *in vitro* binding assays, both peptides show equal affinity at the KISS1R receptor (Kotani, Detheux et al. 2001). Our results demonstrate that the 10 amino acid form of kisspeptin is a potent LH secretagogue *in vivo* in humans.

While there was a clear dose-dependency of LH response to increasing doses of kisspeptin-10 up to 1 μg/kg, the 3 μg/kg (2.3 nmol/kg) bolus dose did not elicit a statistically significant increase when compared to vehicle, and mean and peak LH concentration following this dose was lower than corresponding values following 0.3 and 1 μg/kg. This phenomenon has not been reported before following bolus administration of kisspeptin, but continuous infusion of kisspeptin-10 in primates (Seminara, DiPietro et al. 2006) and repeated subcutaneous administration of kisspeptin-54 in human subjects with hypothalamic amenorrhoea (Jayasena, Nijher et al. 2009) resulted in tachyphylaxis. The kisspeptin receptor KISS1R is known to desensitise rapidly *in vitro* (Pampillo, Camuso et al. 2009), raising the possibility of rapid hypothalamic desensitisation. Whilst such desensitisation is a parsimonious explanation for the decreased response observed here, desensitisation has to be occurring rapidly after kisspeptin-10 administration before maximal stimulation of gonadotropes is achieved. As the fast half-life of LH in healthy men is 18 min (Veldhuis, Iranmanesh et al. 1993) lower peak LH following the 3 μg/kg would suggest that the gonadotrope stimulation was submaximal. An alternative explanation for this observation is that at high concentrations, kisspeptin-10 may stimulate another RF-amide receptor with an inhibitory effect on GnRH or LH secretion. Kisspeptin-10, at nanomolar concentrations, has been shown to bind and activate the gonadotropin inhibitory hormone (GnIH) receptor (GPR 147, NPFFR1) (Oishi, Misu et al. 2010) which is expressed both in the hypothalamus and on gonadotropes (Ubuka, Morgan et al. 2009). Nanomolar plasma concentrations of kisspeptin were achieved after subcutaneous administration of doses of kisspeptin-54 similar to the kisspeptin-10 used in our study (Dhillo, Chaudhri et al. 2007). However, whilst peak plasma kisspeptin-10 concentrations following the 3 μg/kg bolus may have been sufficient to activate GnIH receptors, there is little evidence for a functional role for GnIH in humans and *in vivo* studies in sheep (Clarke, Sari et al. 2008) would suggest that its effects are relatively modest and it is not possible to exclude an effect of GnIH receptor involvement in the infusion studies. The reduced stimulatory efficacy of kisspeptin-10 at high concentrations demands careful dosing in any potential therapeutic applications.

The stimulatory effect of kisspeptin-10 appears to be limited to gonadotropins with no effect on prolactin, cortisol, insulin or TSH. Moreover, there were no adverse events or off-target effects observed at the doses studied here.

4 Effect of sex-steroid feedback and baseline gonadotropin concentration on gonadotropin responses to kisspeptin-10

4.1 Introduction

Administration of kisspeptin-54 stimulates gonadotropin secretion in healthy women (Dhillo, Chaudhri et al. 2007) and women with hypothalamic amenorrhoea (Dhillo, Chaudhri et al. 2007; Jayasena, Nijher et al. 2010) making it a potential provocative test and therapeutic candidate in reproductive endocrine disorders. Studies of kisspeptin in women reported prior to this study being carried out, had used the 54-amino acid peptide, kisspeptin-54 (Dhillo, Chaudhri et al. 2007; Jayasena, Nijher et al. 2009; Jayasena, Nijher et al. 2010). Although Kisspeptin-54 has been shown to be further processed to 14, 13 and 10 amino acid (kisspeptin-10) sequences (Kotani, Detheux et al. 2001) *in vitro,* there is no data to suggest that this occurs *in vivo*. Kisspeptin-10 has the full intrinsic bioactivity of the longer fragments (Kotani, Detheux et al. 2001; Roseweir and Millar 2009), but with a shorter half-life and quicker onset of action (Mikkelsen, Bentsen et al. 2009).

Modulation of the human hypothalamic-pituitary-ovarian axis by sex steroid feedback is well established (Chetkowski, Meldrum et al. 1986; Gill, Sharpless et al. 2002) and is the basis for sex steroid therapy for contraception. Sex steroids exquisitely regulate Kiss1 gene expression positively and negatively (section 1.3.1). During the menstrual cycle, rising estradiol in the follicular phase and the high estradiol and progesterone concentrations in the luteal phase slow pulsatile GnRH secretion and thus gonadotropin secretion. Inhibitory effects of the sex steroids and peptide inhibins at the pituitary gonadotrope also contribute to feedback regulation (Shaw, Histed et al. 2010). Conversely, the menopausal decline in circulating sex steroids and inhibins results in increased GnRH and gonadotropin secretion (Gill, Sharpless et al. 2002). To fully characterise novel modulators of the GnRH axis, it is therefore important to study gonadotropin responses in models of sex steroid excess and deficiency as well as physiological states. Moreover, as there is a wealth of literature relating to the diagnostic and therapeutic use of GnRH (Schally 1999;

Engel and Schally 2007), translational applications of kisspeptin-10 can potentially be guided by comparing kisspeptin-10 mediated LH stimulation with that observed following administration of GnRH.

4.2 Objectives

The objectives of this study were

1) To quantify and establish the time-course of the stimulatory effect of kisspeptin-10 on LH and FSH secretion in women with regular menstrual cycles, in post-menopausal women, and in women taking sex steroid contraceptive preparations.
2) To compare kisspeptin-induced gonadotropin secretion with that following a maximally stimulatory dose of GnRH.

4.3 Methods

4.3.1 Participants

Ten healthy women with regular menstrual cycles, 6 postmenopausal women, and 8 women using progestogen implants or combined oral contraceptive pills for contraception were recruited to the study. All volunteers provided informed written consent and the study was approved by the local research ethics committee (Lothian REC Ref 09/S1101/67). Post-menopausal women had experienced a natural menopause (median months since menopause: 90, range 17-200) and had not taken hormone replacement therapy in the 6 months preceding their study visit. Women using progestogen implants (n=4) had subcutaneous implants containing 68 mg etonogestrel (Implanon, MSD, Hertfordshire, UK) in the arm, inserted between 3 and 24 months previously. Oral contraceptive pills contained 20-30 µg ethinylestradiol and a synthetic progestogen for 21 days of each menstrual cycle, and all women (n=4) had been established on this for a minimum of 6 months prior to study. Sample size calculations were made on the assumption that the effect sizes and variance of gonadotropin responses to kisspeptin-10 would be comparable to previous studies of kisspeptin-54 in women (Dhillo, Chaudhri et al. 2007). Baseline full blood count, renal function, liver function, electrolytes and glucose were within normal limits.

4.3.2 Experimental Protocol

Volunteers attended Edinburgh Clinical Research Facilities in a fasting state and were provided a standardised breakfast. Blood samples were obtained through intravenous cannulae at 15-min intervals for 3 hours before and a further 3 hours after kisspeptin-10 (0.3 μg/kg) administration, as previous studies of kisspeptin-10 have demonstrated that LH returns to baseline within this duration (Chan, Butler et al. 2011; George, Veldhuis et al. 2011). At the end of this 6-hour study period, a 100 μg bolus of GnRH was administered intravenously and four further samples obtained at 15-min intervals - this dose of GnRH and sampling regime is used routinely for clinical assessment of GnRH responsiveness (Cavallo, Richards et al. 1995). Importantly, evidence from studies involving repeated pulsatile administration of GnRH indicate that there is no desensitisation of GnRH response when subsequent pulses are at least 90 minutes apart (Seminara, Beranova et al. 2000). Thus the GnRH response is unlikely to have desensitised to the kisspeptin-10 administered 3h earlier.

LH was measured at all the time points and FSH at 30 min intervals. Premenopausal women not taking steroidal contraception were studied in the follicular phase of menstrual cycle (day 2-7 inclusive) and women taking the combined contraceptive pill were studied 2-7 days after restarting therapy following a routine scheduled 7-day interruption.

4.3.3 Study drugs

Custom-synthesised GMP grade kisspeptin-10 was used for this study. Please see 2.3.1 for details of the study drug

4.3.4 Analysis of hormonal data

Baseline LH and FSH were calculated as the mean of concentrations measured for 3 hours before the administration of kisspeptin-10. Peak LH and FSH responses were identified as the highest concentration measured within 180 minutes of kisspeptin administration. Baseline and peak gonadotropins were compared within each of the study groups using paired Student's *t* tests. ΔLH and ΔFSH post-kisspeptin was calculated as the arithmetic difference between peak and baseline concentrations. To

account for expected variability in baseline gonadotropin concentrations across study groups, percentage increases in ΔLH and ΔFSH over respective baseline values were calculated and one-way ANOVA followed by protected Fisher's test was used to compare groups.

The ratio of ΔLH and ΔFSH following kisspeptin administration to corresponding values observed following GnRH stimulation in each individual was calculated, and groups compared using one way ANOVA followed by Tukey's multiple comparison test.

Additionally, 60-min mean Area-Under-Curve (AUC) pre and post kisspeptin-10 bolus were calculated by trapezoid integration. To account for inter-individual variability mean serum gonadotropin concentration observed 60 minutes before the administration of kisspeptin-10 was deducted from observed gonadotropin concentrations. Data are presented as mean±SEM.

Data not normally distributed were log-transformed prior to statistical analysis. A two-sided $P < 0.05$ was regarded as statistically significant. The statistical software package Minitab 16 (Minitab Ltd, Coventry, UK) was used.

4.4 Results

Baseline age, weight, BMI, LH, FSH and estradiol of subjects are summarised in Table 4.1. Post-menopausal women had significantly lower estradiol and higher gonadotropin concentrations, and were older.

No adverse events were observed.

Table 4.1: Baseline Characteristics of women undergoing intravenous kisspeptin-10 stimulation (0.3 μg/kg). Data shown as mean±SEM and analysed by one-way ANOVA followed by Fisher's test. [a, b] Groups that do not share a letter show significant difference. Post-menopausal women had significantly lower estradiol and higher gonadotropin concentrations, and were older.

	Follicular	Postmenopausal	Progesterone Implant	COCP	P value
n	10	6	4	4	
Age (years)	33.4±2.8	58.2±1.8 [a]	26.3±1.1	26.3±1.8	<0.0001
BMI (kg/m^2)	25.3±1.2 [a]	25.1±1.1 [a]	23.4±1.4 [a]	27.1±2.4 [a]	0.545
E (pmol/L)	133.7±18.7 [a]	<50 [b]	98.0±16.0 [a, b]	<50 [b]	0.008
LH (IU/L)	8.0±1.4	29.3±4.7[a]	5.7±0.9	2.63±0.8	<0.0001
FSH (IU/L)	6.3±0.8	65.7±8.8 [a]	5.1±0.3	6.2±2.6	<0.0001

4.4.1.1 Kisspeptin-10 stimulates LH secretion in women

Acute intravenous administration of kisspeptin-10 resulted in a significant increase in LH in all groups of women. In women in the follicular phase, LH rose from 6.3±1.2 to 9.4±1.3 IU/L (P=0.006, Figure 4.1). Peak LH serum concentrations were achieved by 30 min, declining thereafter to reach pre-administration levels by 180 min. Postmenopausal women also responded to kisspeptin-10 administration, with an increase in serum LH from 35.3±2.8 to 44.7±3.4 IU/L (P=0.005). Women with etonogestrel implants and women taking ethinyl estradiol plus a progestogen in the combined contraceptive pill had the lowest pre-treatment LH concentrations but also showed a significant rise in LH in response to kisspeptin-10 (4.6±0.2 to 7.5±0.9 IU/L, P=0.02; 2.3±0.9 to 3.7±1.4 IU/L P<0.001 respectively Figure 4.1). Analysis of 60-min AUC of LH showed similar results, as summarised in figure 4.2.

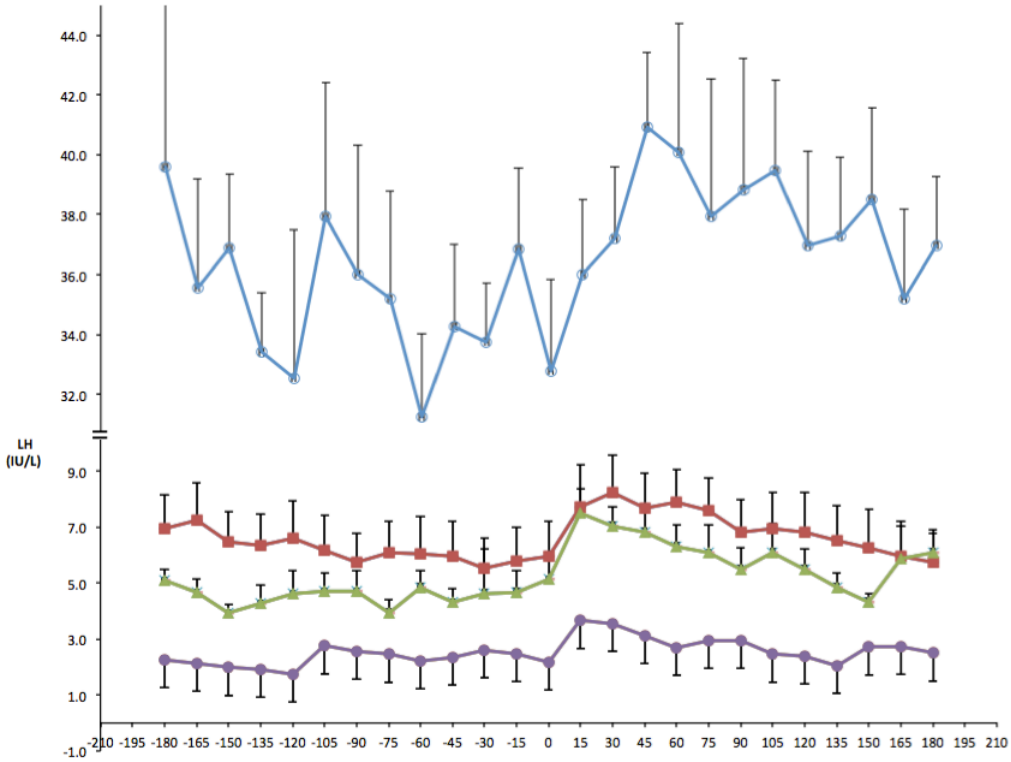


Figure 4.1: LH responses to kisspeptin-10 in women in follicular phase (red squares, n=10), progestogen implant (green triangles, n=4), women on combined oral contraceptives (closed purple circles, n=4) and postmenopausal women (blue open circles, n=6).

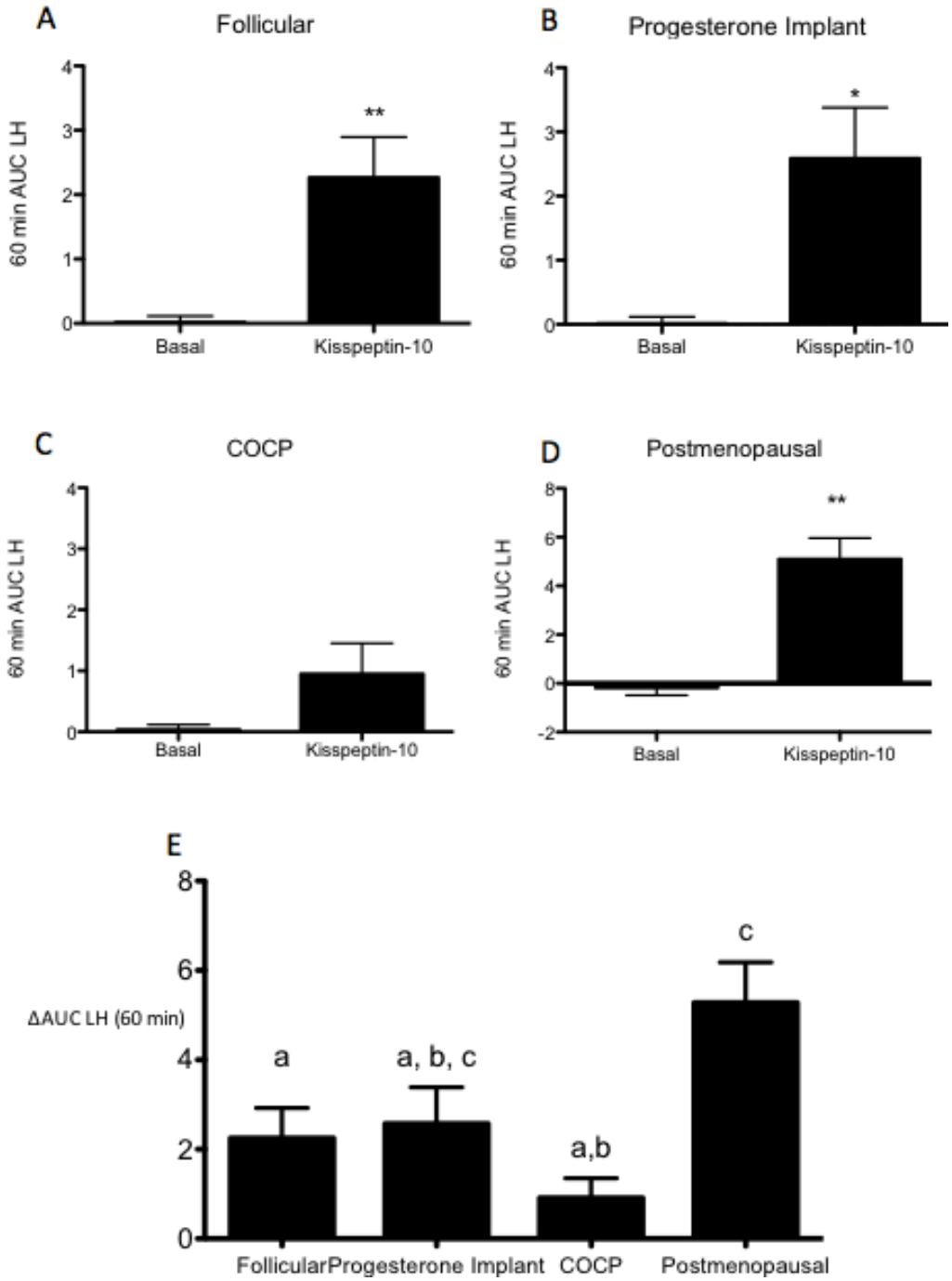


Figure 4.2. LH responses to intravenous kisspeptin-10 in women. A-D, AUC of LH 60 minute before (basal) and after kisspeptin-10 stimulation in women. E, Δ AUC LH (60-min) following kisspeptin administration in the four groups of women studied. Groups that do not share a letter show significant statistical difference with each other (One- way ANOVA followed by Tukey's test). Δ AUC LH in postmenopausal women were higher than that of women in follicular phase and women treated with combined oral contraceptive pills.

4.4.1.2 Comparison between kisspeptin and GnRH LH responses

Following 100 µg GnRH bolus, serum LH increased significantly in women in the follicular phase (6.3±1.1 to 35.7±7.2 IU/L, P<0.001), postmenopausal women (35.3±2.8 to 94.0±10.3 IU/L, P<0.001) and in women with etonogestrel implants (4.6±0.2 to 26.5±5.2 IU/L, P=0.002). Women on the combined contraceptive pill showed a smaller response that did not reach statistical significance (2.3±0.9 to 7.2±2.1 IU/L P=0.17 Figure 4.3).

Peak LH observed following kisspeptin-10 was 30.3±4.3 % of the GnRH induced LH peak in women studied in the follicular phase, 49.3±4.8 % in postmenopausal women, 30.0±4.1 % in women with etonogestrel implants and 66.7±26.0 % in women on the combined contraceptive pill.

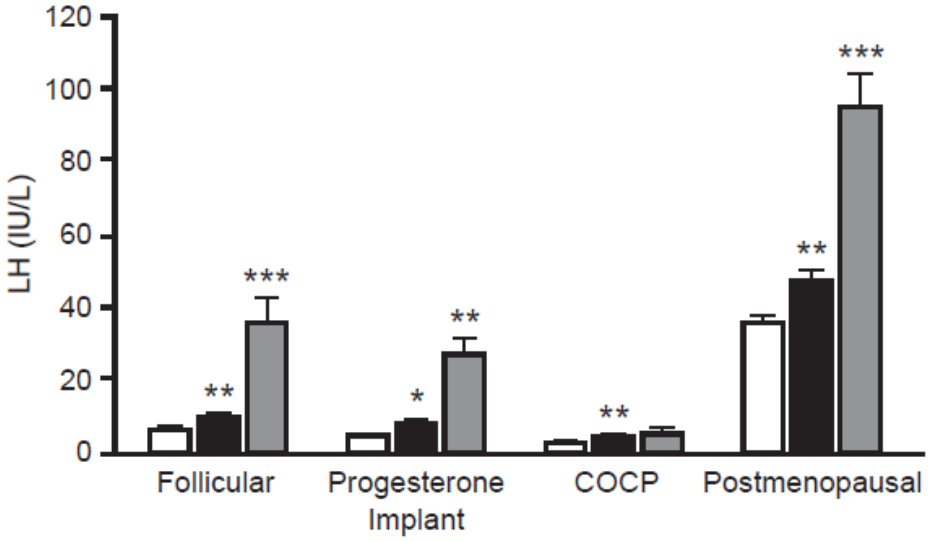
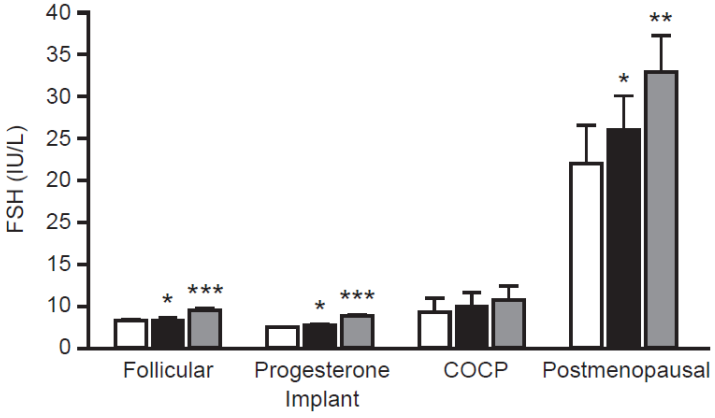


Figure 4.3: Comparison of Peak LH response to kisspeptin-10 and GnRH. Serum LH at baseline (white column), peak LH following kisspeptin administration (black columns) and peak LH following GnRH (100 µg) administration (grey columns). Mean±sem,* P<0.05, **P<0.01, *** P<0.001 vs. baseline.

4.4.1.3 Kisspeptin-10 stimulates FSH secretion in women

Serum FSH also increased following kisspeptin-10 administration, from 3.2±0.4 to 3.4±0.3 IUL/L (P=0.04) in women in follicular phase, from 21.8±4.9 to 25.7±4.4 IU/L (P=0.04) in postmenopausal women and from 2.4±0.1 to 2.6±0.1 IU/L (P=0.02) in women with etonogestrel implants. FSH however remained unchanged in women taking the combined contraceptive pill (4.1±1.8 to 4.7±2.0 IU/L P=0.17 Figure 4.4). Analysis of 60-min AUC showed significant increase in FSH only in the post-menopausal group (Figure 4.5).

In response to 100 µg GnRH, serum FSH increased in women in the follicular phase (3.2±0.4 to 4.4±0.5 IUL/L, P<0.001), in postmenopausal women (21.8±4.9 to 32.6±4.6 IU/L P=0.008), and in women with etonogestrel implants (2.4±0.1 to 3.6±0.2 IU/L P=0.001). As with LH, women on the combined contraceptive pill showed no significant change in FSH in response to GnRH (4.1±1.8 to 5.6±1.9 IU/L P=0.22) (Figure 4.4). Peak FSH observed following kisspeptin-10 was 73.9±2.6 % of the GnRH-induced LH peak in women studied in the follicular phase, 82.4±11 % in postmenopausal women, 72.4±3.4 % in women with etonogestrel implants and 75.0±20.4 % in women on the combined pill.



Figures 4.4: Comparison of Peak FSH response to kisspeptin-10 and GnRH. Serum FSH at baseline (white column), peak FSH following kisspeptin administration (black columns) and peak FSH following GnRH administration (grey columns). Mean±sem,* P<0.05, **P<0.01, *** P<0.001 vs. baseline.

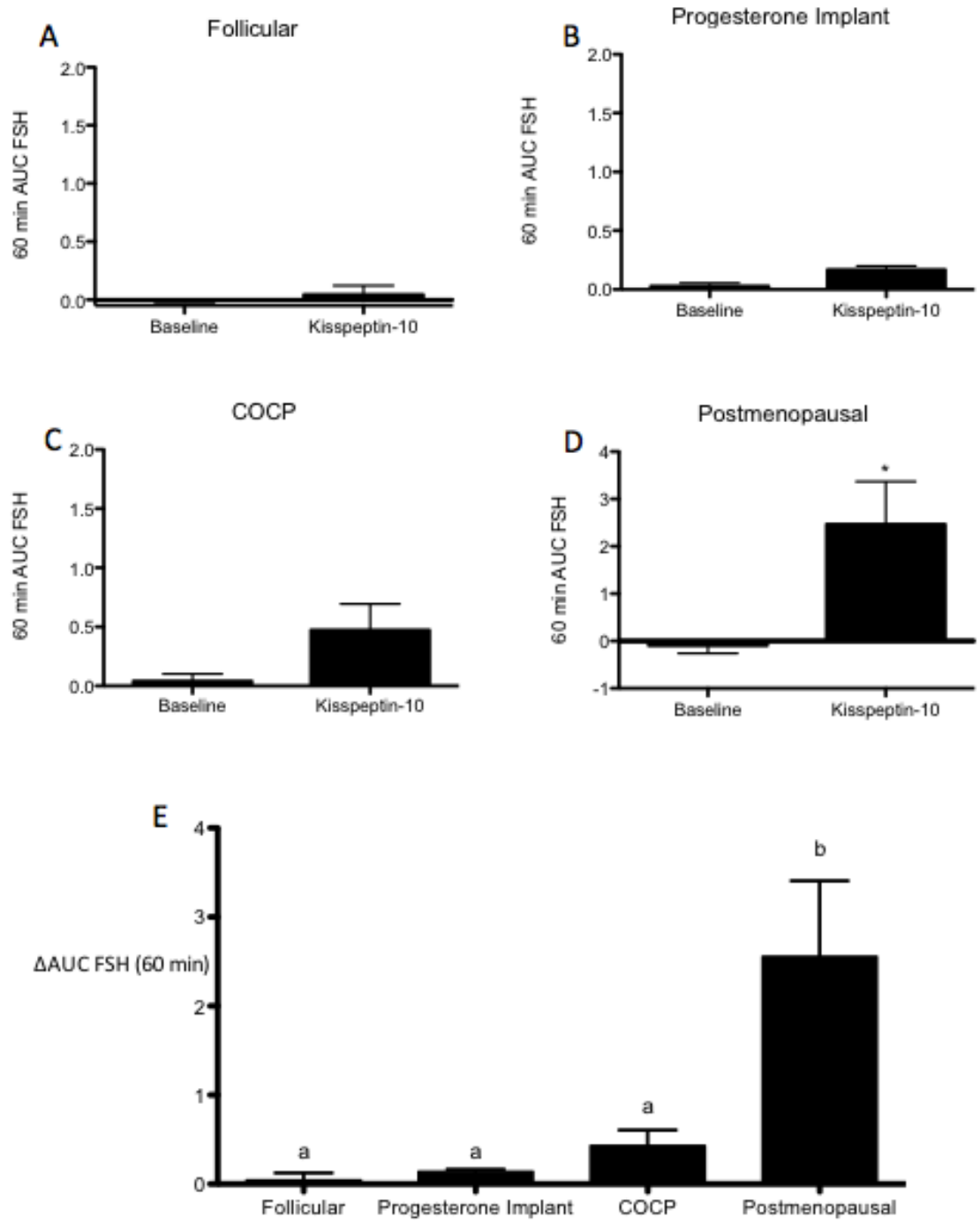


Figure 4.5. FSH responses to intravenous kisspeptin-10 in women A-D, AUC of FSH 60 minute before (basal) and after kisspeptin-10 stimulation. E, Δ FSH (60-min) following kisspeptin in the four groups of women studied. * P<0.05. Groups that do not share a letter show significant statistical difference (One- way ANOVA followed by Tukey's test). Postmenopausal women had higher LH than the other groups studied.

4.4.1.4 Incremental gonadotropin rise in postmenopausal women and women exposed to sex steroids.

The incremental rise in LH and FSH in response to kisspeptin-10 was compared between the groups studied. Both ΔLH and ΔFSH as a proportion of baseline LH/FSH were similar in the four study groups (P=0.07 and 0.095 respectively, Figure 4.6A and B). ΔLH and ΔFSH following GnRH administration were also similar in all groups (P=0.95 and 0.93).

The ratio of gonadotropin response following kisspeptin-10 administration to that observed following GnRH administration also showed no statistically significant difference between study groups for both LH (P=0.28) and FSH (P=0.32).

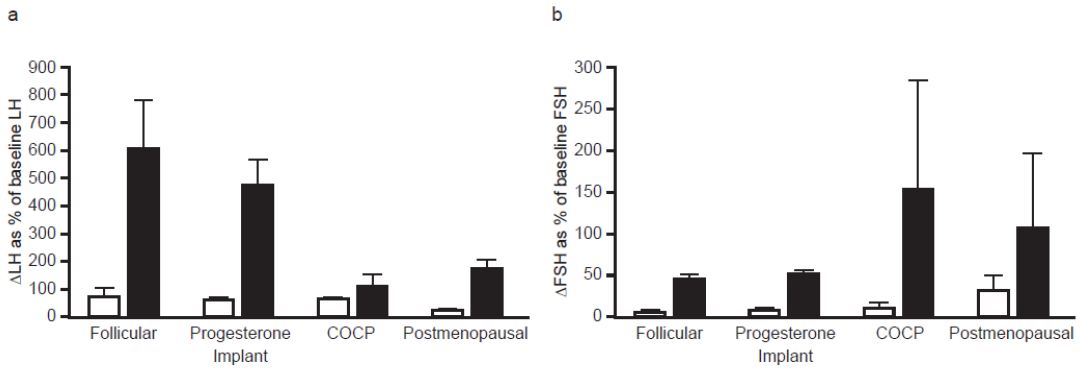


Figure 4.6: Gonadotropin responses to kisspeptin-10 and GnRH. A: ΔLH as percentage of baseline LH following 0.3 μg/kg kisspeptin-10 (white columns) and 100 μg GnRH (black columns) administered intravenously to premenopausal women in follicular phase of menstrual cycle ('Follicular', n=10), women with progesterone implant ('Progesterone Implant, n=4), women on combined oral contraceptives ('COCP', n=4) and postmenopausal women (n=6). B: ΔFSH as percentage of baseline FSH following 0.3 μg/kg kisspeptin-10 (white columns) and 100 μg GnRH (black columns) administered intravenously. No significant variance was observed (P= 0.07 for LH and 0.095 for FSH on ANOVA). Note the difference in scale between the two column graphs.

4.5 Discussion

These data demonstrate that kisspeptin-10 has a stimulatory effect on LH and FSH secretion in women. These responses are preserved in postmenopausal women and in women exposed to steroidal contraceptives.

Our results, in keeping with previous studies (Dhillo, Chaudhri et al. 2005; Dhillo, Chaudhri et al. 2007; George, Veldhuis et al. 2011), demonstrate that kisspeptin preferentially stimulates LH over FSH, an observation of significance to consider in potential therapeutic applications. An explanation is that the magnitude and/or duration of GnRH release in response to kisspeptin may not be sufficiently large enough to stimulate FSH secretion, which is known to have a constitutive component which is less dependent on GnRH pulsatility (Padmanabhan, McFadden et al. 1997). This observation is consistent with earlier studies using a lower dose (25 μg) of GnRH which elicited isolated LH secretion (Franchimont, Becker et al. 1974). Another plausible explanation for preferential secretion of LH is kisspeptin mediated increase in GnRH pulse frequency. The frequency of GnRH pulsatility has been demonstrated to selectively regulate gonadotropin subunit gene transcription. High pulse frequency preferentially increases α and LH-β gene expression whilst lower pulse frequency increases FSH-β gene transcription (Dalkin, Haisenlender et al. 1989; Haisenleder, Dalkin et al. 1991; Kaiser, Jakubowiak et al. 1997). A decrease in GnRH pulse frequency has also been reported in animal models administered kisspeptin antagonists (Li, Kinsey-Jones et al. 2009).

In the present study, we have also demonstrated that maximal kisspeptin-10 -induced LH secretion is less than that observed with a maximally stimulatory dose of GnRH, irrespective of the steroidal milieu. We compared the magnitude of secretion of LH following administration of a maximally stimulatory dose of kisspeptin-10 with that observed following administration of a maximally stimulatory dose of GnRH (100 μg intravenous bolus). LH secretory response to GnRH was higher than the response to kisspeptin-10 in all groups. Kisspeptin independent GnRH secretion has been demonstrated in *Kiss1* and *GPR54* knock-out rodents models (Chan, Broder-Fingert et al. 2009) and kisspeptin antagonists, unlike GnRH antagonists, do not lower LH to sub-basal levels (Roseweir, Kauffman et al. 2009; Millar, Roseweir et al. 2010). Our

observations are consistent with these studies and the notion that while the principal mode of action of kisspeptin is GnRH mediated (Oakley, Clifton et al. 2009), there is a kisspeptin independent component of GnRH secretion. This suggests potential therapeutic application of kisspeptin and its agonist analogs will primarily be in areas where modest rises in serum concentration of gonadotropins are desirable, such as induction of mono-ovulation rather than super-ovulation prior to IVF. Similarly, kisspeptin antagonists would, in principle, be an attractive proposition in the treatment of hormone dependant diseases (e.g. endometriosis) where a partial lowering rather than complete suppression of sex steroids would be therapeutically advantageous.

In studies of kisspeptin-10 effects in men, we have demonstrated a doubling of LH from baseline following the administration of a bolus dose of kisspeptin-10 (chapter 3). LH responses in women studied here are smaller, with peak values being 50-60% higher than baseline. Nevertheless, the magnitude of LH stimulation observed in this study (3.1±0.7 IU/L) during the follicular phase was markedly higher than that observed following subcutaneous administration of kisspeptin-54 (0.12 ± 0.17 IU/L) to women in similar menstrual phase at comparable doses (0.23 nmol/kg of kisspeptin-10 vs. 0.4 nmol/kg kisspeptin-54) (Dhillo, Chaudhri et al. 2007). Moreover, kisspeptin-10 in our studies elicited peak LH responses within 30 minutes of intravenous administration, compared to peak values observed more than 2 hours following subcutaneous kisspeptin-54 administration. Whilst these observations might merely reflect the differences in pharmacokinetics following administration through intravenous and subcutaneous routes, they are also in keeping with rodent data showing a faster onset of action and shorter half-life for kisspeptin-10 (Mikkelsen, Bentsen et al. 2009).

There are further physiological inferences to be made from our studies. There is an increase in *Kiss1* expression and corresponding increase in LH secretion in ovariectomised rodents, monkeys and sheep, which is reversed by treatment with sex steroids (Rometo, Krajewski et al. 2007; Smith, Clay et al. 2007; Navarro, Gottsch et al. 2009). Histological analysis of the human hypothalamus has demonstrated an increase in *KiSS1* expression in the infundibular nucleus following menopause

(Rometo, Krajewski et al. 2007), consistent with findings in animal models (Rometo, Krajewski et al. 2007; Smith, Clay et al. 2007; Navarro, Gottsch et al. 2009). The robust response to kisspeptin-10 in postmenopausal women in this study suggests that although hypothalamic *KISS1* gene expression is increased with hypertrophy of kisspeptin neurons (Rance and Uswandi 1996; Rance 2009), there is residual capability for GnRH neurons to respond to kisspeptin. This suggests that endogenous kisspeptin secretion in the postmenopausal state is lower than that required to elicit maximal GnRH response. This suggests that factors other than sex steroids might regulate KiSS1 expression.

We have demonstrated stimulatory effects of kisspeptin-10 in women exposed to excess steroid, in steroid-deficient women and in steroid-sufficient women. As kisspeptin's action are predominantly GnRH mediated (Oakley, Clifton et al. 2009), these observations support the notion that sex steroid feedback in women occurs both at the level of the pituitary and at the hypothalamus (Crowley and Mcarthur 1980; Hall, Taylor et al. 1994; Pitteloud, Dwyer et al. 2008). The relatively smaller response in women taking combined estogen/progestogen contraceptive is also consistent with an inhibitory negative feedback effect of estrogen at the pituitary level (Shaw, Histed et al. 2010). The lower ΔLH observed here following GnRH administration in postmenopausal women and in women taking combined the contraceptive pill (in comparison to women in the follicular phase) are also consistent with previous clinical studies of GnRH (Thomas, Cardon et al. 1973; Franchimont, Becker et al. 1974; Cohen and Katz 1979; Geller and Scholler 1980).

While it is clear that GnRH secretion is inhibited by endogenous and exogenous estrogen (Herbison 1998; Couse and Korach 1999; Gill, Sharpless et al. 2002), the pathways involved remain unclear. The absence of expression of estrogen receptor alpha (ESR1) in GnRH neurons (Shivers, Harlan et al. 1983; Herbison and Theodosis 1992) suggest another hypothalamic neuronal population is required to mediate this feedback effect. Kisspeptin neurons express ERα and are strong candidates for this role (Tena-Sempere 2005; Rance 2009) and the presence of detectable LH response to kisspeptin in women taking exogenous steroids lends indirect support to this notion that steroid input is not received directly at the level of the GnRH neuron.

Had this been the case, kisspeptin would not have been able to stimulate GnRH secretion.

Our findings are at variance with the recently published report that kisspeptin-10 does not stimulate LH or FSH secretion in women studied in the follicular phase when administered as intravenous boluses of 1, 3 and 10 nmol/kg (Jayasena, Nijher et al. 2011). These doses used by Jayasena et al were 5 to 50 times higher than the 0.23 nmol/kg dose that we have used in the present study and similar to those we have previously shown to result in a reduced LH secretion in healthy men (chapter 3). It has to be noted that Jayasena and colleagues only tested LH responses in women at the higher doses (1,3 and 10 nmol/kg) whereas the men were administered an additional lower dose (0.3 nmol/kg) at which they had significant LH and testosterone responses (Jayasena, Nijher et al. 2011). Interestingly, an absence of significant testosterone response at 3 and 10 nmol/kg in men studied by Jayasena and colleagues, further suggesting that the apparent lack of effect observed in the study is a function of the dosage employed. There are a number of potential explanations for the diminished LH response with higher doses of kisspeptin-10, including rapid desensitization and stimulation of inhibitory RF-amide receptors, as outlined in Chapter 3. The present study also has a higher number of women studied in the follicular phase (n=10 in the present study vs. 5) providing greater statistical power to detect small but significant changes. Moreover, the present study employed a 180 min baseline LH profile enabling comparisons of LH secretion before and after kisspeptin-10 administration in the same individual. This approach provides the opportunity to obtain a more accurate view of baseline secretion in gonadotropin concentrations both within and between individuals, thereby increasing the sensitivity to detect small changes. However, it has to be noted that the increases in LH observed in these studies are small and the physiological significance of such small increases in LH are yet unclear. Therefore, from the perspective of translational clinical applications, the present findings are consistent with the findings reported by Jayasena and colleagues (Jayasena, Nijher et al. 2011). The modest increase in LH observed in the present study and the lack of LH stimulation observed by Jaysena et al raises the possibility that kisspeptinergic stimulation of LH may not be applicable to clinical care. Whilst this is indeed the parsimonious

conclusion, it has to be noted that the stimulation of LH observed in men following continuous exposure to kisspeptin-10 (Chapter 5) is considerably higher than that observed following the administration of intravenous bolus doses (Chapter 3). The translational therapeutic/diagnostic value of kisspeptin-10 in women can only be fully understood with prolonged administration studies using a range of doses.

Our study did not involve the measurement of kisspeptin immunoreactivity as the plasma half-life of kisspeptin-10 is very short and evaluation would require higher frequency of sampling. Moreover, in mass-spectroscopic assays of serum samples from our recent studies of kisspeptin-10 involving administration of higher doses in men, concentrations of kisspeptin-10 and its cleavage products (kisspeptin 9, 7 or 4) were below the detection limit (1 ng/ml) (Chapter 5).

In conclusion, kisspeptin-10 stimulates LH release in women irrespective of prevailing steroid milieu. We have also demonstrated that kisspeptin induced LH secretion in women is only a proportion of that observed with a maximally stimulatory dose of GnRH.

5 Continuous intravenous infusion of kisspeptin-10 elicits sustained LH and testosterone secretion in men.

5.1 Introduction

Appropriate modulation of GnRH pulsatility is integral to the development and maintenance of adult reproductive function (see section 1.1.3). Alterations in GnRH pulsatility and thus LH secretion are a feature of a number of reproductive disorders. Individuals with some forms of male hypogonadism (Veldhuis, Keenan et al. 2009) and hypothalamic amenorrhea (Gordon 2010) show decreased pulse frequency while women with polycystic ovarian syndrome show an increase (Blank, McCartney et al. 2006). However, neuroendocrine mechanisms underpinning GnRH pulse generation are yet to be fully delineated (Krsmanovic, Hu et al. 2009) as summarised in chapter 1.1.3). Experimental animals exposed to kisspeptin antagonists demonstrate decreased LH pulsatile secretion suggesting that kisspeptin modulates GnRH pulse frequency (Li, Kinsey-Jones et al. 2009).

Continuous exposure to kisspeptin has been demonstrated to lead to desensitisation of kisspeptin response. In juvenile male rhesus monkeys infused with kisspeptin-10 (100 μg/hr, approximately 30 μg/kg/hr), an initial surge in LH response was observed in the first three hours but declined thereafter. Subsequent serum LH concentrations were indistinguishable from those observed in control animals infused vehicle (Seminara, DiPietro et al. 2006). Similar observations have been made in adult male rhesus monkeys (Ramaswamy, Seminara et al. 2007) and rodents (Roa, Vigo et al. 2008). In women with hypothalamic amenorrhea receiving subcutaneous Kp-54 (37 μg/kg; 6.4 nmol/kg) twice daily for two weeks, potent increases in serum LH and FSH seen on day one were significantly diminished at the end of the study (Jayasena, Nijher et al. 2009).

Experimental animals exposed to kisspeptin antagonists demonstrate decreased LH pulsatile secretion suggesting that kisspeptin modulates GnRH pulse frequency (Li, Kinsey-Jones et al. 2009) and infusion of kisspeptin-10 at 4 μg/kg/hr elicited

sustained increase in LH secretion with suggestion of an increase in LH pulse frequency.

5.2 Objectives

The objectives of the study were:

- To investigate the effects of infusion of kisspeptin-10 on LH pulsatility.
- To determine if continuous infusion of a maximally stimulatory dose of kisspeptin-10 induces tachyphylaxis/desensitisation of LH response.
- To compare the magnitude of maximal kisspeptin-10 mediated LH secretion with that following a maximally stimulatory dose of GnRH.
- To determine the effect of continuous exposure to kisspeptin-10 on LH pulse frequency.

5.3 Materials and methods

5.3.1 Volunteers

Four healthy men took part in this study. All volunteers provided informed written consent and the study was approved by a local research ethics committee (Lothian REC Ref 09/S1101/23). The age of healthy male participants was 35.6±3.4 (mean±SEM) and body mass index was 26.1±0.8 kg/m^2, all of whom had a minimum bilateral testicular volume of 40 ml, normal physical examination and normal secondary sexual characteristics. Baseline full blood count, renal function, liver function and electrolytes were within normal limits.

5.3.2 Protocol

5.3.2.1 Effects of prolonged high-dose infusion of kp-10 on LH pulsatility in healthy men

Following overnight fast, 4 volunteers attended our clinical research facility for a 34-hour supervised stay. Blood samples were collected at 10 min intervals for two 12 hour periods on consecutive days and hourly overnight. After 9 hours of baseline sampling, 3µg/kg of kisspeptin-10 (the highest dose previously investigated) was

administered as an iv bolus and a continuous iv infusion of kisspeptin-10 (Crono PCA, Cane, Rivoli, Italy) commenced 90 min later at 4μg/kg/hr. This infusion rate, maintained for 22.5 hrs, was selected to aim to give a comparable hourly dose to the maximally effective bolus doses, bearing in mind the expected short half-life of kisspeptin-10. A 100 μg/kg iv bolus of GnRH was administered 60 min before the end of the infusion.

Serum LH during the 9 hour baseline frequent sampling period was compared with the corresponding 9 hour frequent sampling period on the second day of the study during the iv infusion of kisspeptin-10. In addition, we compared the mean LH values observed in the initial 90 min of kisspeptin-10 infusion with the mean LH values observed in the final 90 min of infusion before GnRH administration to identify any potential tachyphylaxis.

Comparisons were made between mean LH over the 60 min before GnRH administration and the peak LH 30 min after. Serum testosterone was analyzed at 30-min intervals during the two 9-hour frequent sampling periods before and during the infusion.

All subjects were provided with standardised meals throughout the study and a period of overnight fast maintained between 2200 and 0900. Full blood count, electrolytes, liver function and renal function were checked at the beginning of the study, 24 hours later and at the end of the study.

5.3.2.2 Effects of low dose infusion of kp-10 on LH pulsatility in healthy men

Four healthy men attended our clinical research facility for two visits five days apart for 10-min blood sampling. At the first visit, baseline LH pulsatility was assessed over a 9-hour period. During the second visit, an intravenous infusion of kisspeptin-10 was administered for 9 hours at 1.5μg/kg/hr after an hour of baseline sampling. Subjects were provided with standardised meals during the study visits.

5.3.3 Study Drugs

GMP kisspeptin-10 was prepared as 0.2mg/ml solution as described in chapter 3.3.1 and GnRH was sourced from Sanofi-Aventis (Relefact, Sanofi Aventis, Frankfurt, Germany).

5.3.4 Statistical Analysis

LH pulses were identified using a deconvolution algorithm using cluster analysis with 93% sensitivity and specificity on blinded data, and basal, pulsatile and total LH secretion calculated (Liu, Keenan et al. 2009). Paired Student's *t* test was used to assess changes in pulse frequency and changes in mean LH, FSH and testosterone concentrations. Data are presented as mean±sem. A two-sided P <0.05 was regarded as statistically significant for all analyses. The statistical software package Minitab 16 (Minitab Ltd, Coventry, UK) was used.

5.4 Results

5.4.1.1 Effects of prolonged high-dose infusion of kp-10 on LH pulsatility in healthy men

Infusion of 4-μg/kg/hr kisspeptin-10 resulted in sustained increases in LH concentration. LH profiles observed in the 4 individual study subjects are represented in Fig. 5.1. Bolus injection of 3μg/kg kisspeptin-10 resulted in an increase in LH to 13.6 ±1.7 IU/L (P <0.05 vs. baseline) at 30 min. Continuous kisspeptin-10 infusion (4 μg/kg/hr) resulted in a further increase in LH secretion in all subjects which was sustained throughout the 22.5 hours of infusion. Mean LH during kisspeptin-10 infusion was 20.9±4.9 vs. 5.5±0.8 IU/L over the 9 hours pre-treatment period (P <0.05, Fig 5.1).

Mean LH concentrations in the final 90 min of infusion (23.9±6.8 IU/L) were comparable to that in the first 90 min of infusion (16.3±2.8 IU/L, P=0.34). These data therefore do not indicate tachyphylaxis of the LH response to kisspeptin-10 administration at this dose and duration of exposure.

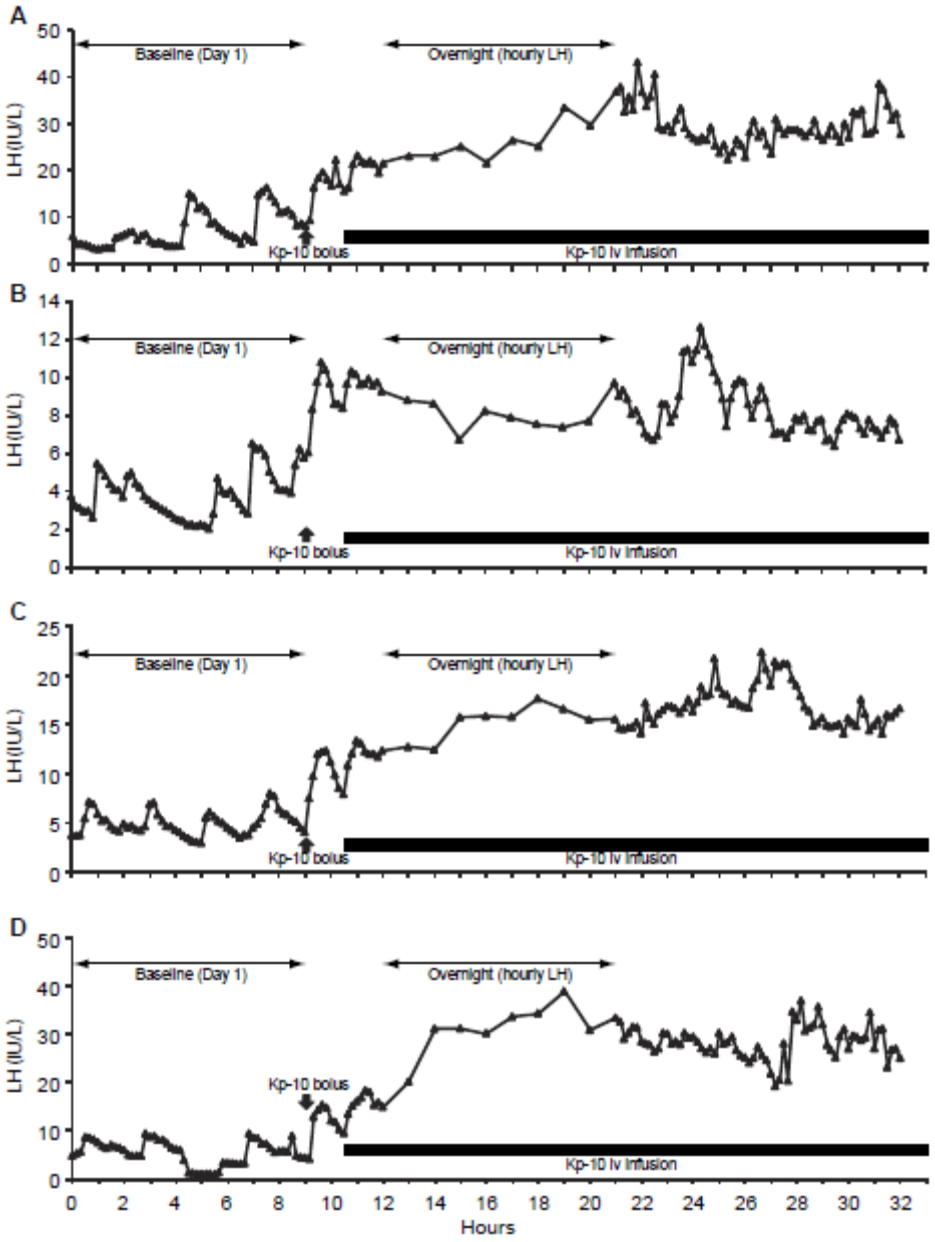


Figure 5.1. Serum LH profiles of four individual subjects receiving 4 µg/kg/hr kisspeptin-10 infusion (black bar) after 9 hour baseline sampling and 3µg/kg iv bolus administered at 9 hours (black arrow). Serum samples were obtained at 10 min intervals except overnight when samples were obtained hourly. Note the difference in scale between individual subjects.

Intravenous administration of 100 μg bolus of GnRH 60 min before the end of the kisspeptin-10 infusion increased LH further with a peak of 89.3±6.1 IU/L. This stimulatory effect of GnRH was at least 2.5-fold greater in all subjects than the peak LH observed with the iv bolus of kisspeptin-10 (13.6±1.7 IU/L, $P < 0.001$) and the peak LH achieved during kisspeptin-10 infusion (28.7±7.0 IU/L, $P < 0.01$).

Analysis of LH pulse frequency during the 9-hour baseline sampling and the corresponding 9-hour period on the second day of infusion was performed. Characteristic pulsatile LH secretion was observed in all 4 subjects at baseline, but was not well defined during kisspeptin-10 infusion. Nevertheless, deconvolution analysis showed an in increase in pulse frequency in three subjects (from 0.4 to 0.6, 0.6 to 0.8 and 0.6 to 1.2 pulses/hr, Fig. 5.1A to 5.1C respectively) while one subject showed a slight decrease from 0.7 to 0.6 pulse/hr giving a mean pulse frequency of 0.6±0.1 pulses/hr at baseline and 0.8±0.2 during kisspeptin-10 infusion (ns).

Although FSH showed an increase in all subjects during infusion of 4 μg/kg/hr kisspeptin-10, this was not statistically significant (2.7±0.8 to 5±2.0 IU/L, $P = 0.17$, Fig. 5.2 B) whereas testosterone concentration significantly increased from 16.6±2.4 to 24.0±2.5 nmol/L ($P < 0.001$, Fig. 5.2 C).

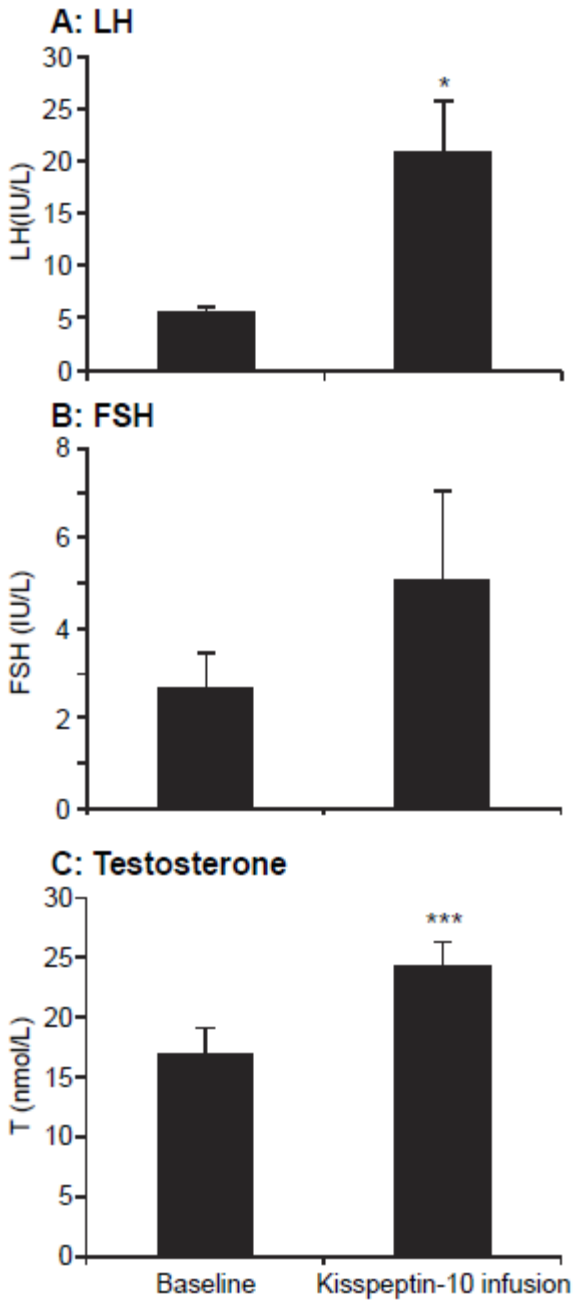


Figure 5.2. Gonadotropin and testosterone response to infusions of 4 µg/kg of kisspeptin-10 in healthy men (n=4). A: Mean serum LH at baseline and during the final hour of kisspeptin-10 infusion. B: Mean serum FSH before and during kisspeptin-10 infusion. C: Mean serum testosterone before and during kisspeptin-10 infusion. Error bars represent SEM. * $P<0.05$, *** $P<0.001$.

5.4.1.2 Effects of low dose infusion of kp-10 on LH pulsatility in healthy men

Mean LH increased significantly from 5.2±0.8 IU/L at baseline to 14.1±1.7 IU/L during kisspeptin-10 infusion (P <0.01, n=4, Fig 5.3). LH showed a steady increase throughout the course of the infusion, increasing from 7.2±2.3 IU/L in the first 90 min of infusion to 20.4±3.5 IU/L in the last 90 min (P <0.05, Fig. 5.3).

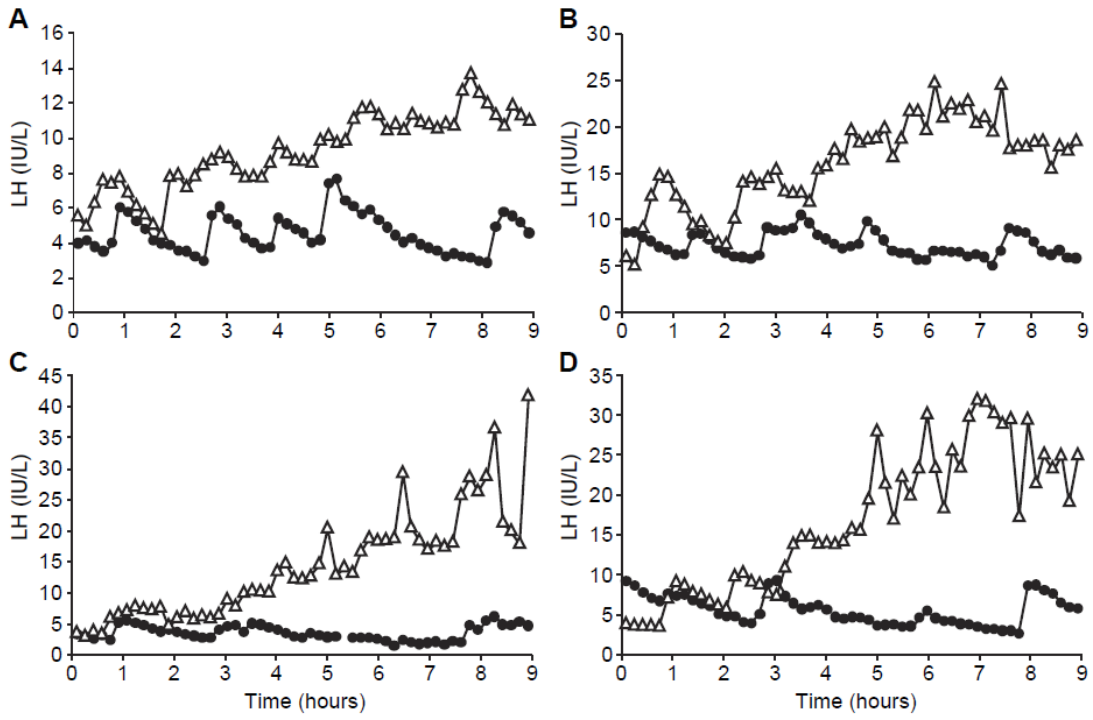


Figure 5.3: Gonadotropin response to 9-hour intravenous infusion of 1.5 µg/kg kisspeptin-10 (n=4). A to D: LH profiles from individual subjects during baseline (closed circles) and kisspeptin-10 infusion (open triangles) visits. Note the difference in scale between individual subjects.

Deconvolution analysis demonstrated an increase in LH pulse frequency in all subjects, with a mean increase from 0.7±0.1 to 1.0±0.2 pulses/hr (P = 0.01) during kisspeptin-10 infusion at 1.5 µg/kg/hr (Fig. 5.4). Secretory mass of LH per pulse also increased in all subjects during the infusion, from 3.9±0.4 to 12.8±2.6 IU/L (P <0.05,

Fig 5.4). Pulsatile LH secretion increased as a proportion of total LH secretion in all subjects from 41.3±5.7% to 69.5±5.9% during the infusion.

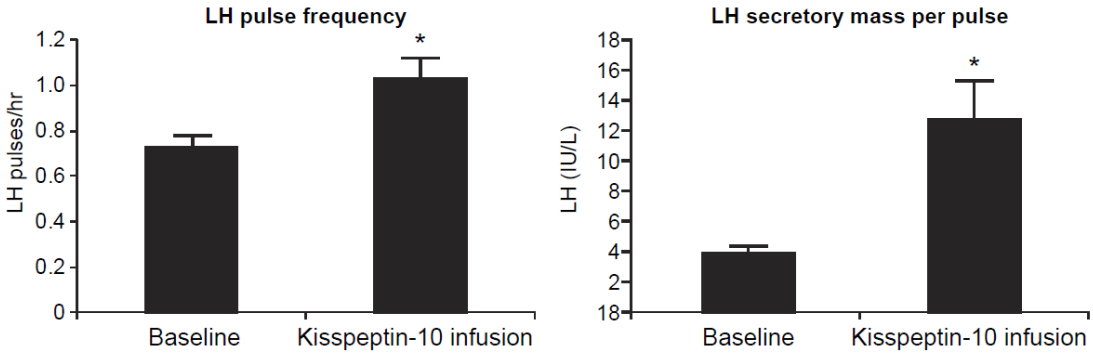


Figure 5.4: Mean LH pulse frequency and secretory mass of LH per pulse during baseline and kisspeptin-10 infusion visits. Error bars represent SEM. * P<0.05.

Serum FSH also increased progressively during the lower dose kisspeptin-10 infusion. FSH observed during the final 90 minutes of the infusion (6.6±0.8 IU/L) showed a significant increase from the first 90 min (3.8±0.8 IU/L $P < 0.05$) of the infusion and baseline (3.9±0.7 IU/L $P < 0.05$).

5.5 Discussion

Continuous infusion of kisspeptin-10 elicited sustained LH secretion without any evidence of rapid desensitisation. Tachyphylaxis of the GnRH/LH system during continuous infusion of kisspeptin-10 has been demonstrated in primate models, with LH returning to baseline a few hours after an initial rise (Seminara, DiPietro et al. 2006; Ramaswamy, Seminara et al. 2007). Despite continually infusing kisspeptin-10 for 22.5 hours, we found no such desensitisation and indeed LH secretion tended to increase progressively. This is possibly a function of the dose of kisspeptin-10 used in our study being lower than that used in the primate studies –i.e. 4 µg/kg/hr in the present study vs. approximately 30-40 µg/kg/hr in primates (Seminara, DiPietro et al. 2006; Ramaswamy, Seminara et al. 2007). Tachyphylaxis of LH response has been seen during the twice-daily administration of kisspeptin-54 to women with hypothalamic amenorrhea, but this occurred over a much longer time period, i.e. two

weeks (Jayasena, Nijher et al. 2009). One possibility, albeit with only indirect evidence, is diminished pituitary sensitiveness to increased GnRH secretion triggered by kisspeptin-10. In ovine models infused kisspeptin-10, LH concentrations diminished after an initial surge, while GnRH concentrations (measured directly from portal circulation) did not show a similar decline – suggesting a pituitary locus for desensitisation (Messager, Chatzidaki et al. 2005). However, responsiveness to GnRH was preserved in the primates where kisspeptin infusions lead to desensitisation and in the present study, which is not consistent with this observation in sheep. Finally, it is possible that interspecies difference do exist between rhesus monkeys and humans – as illustrated by a lack of effect reported to 200 μg/hr (20 μg/kg/hr) kisspeptin-10 in contrast to observations in the present study.

Another key observation is the relative constancy of serum LH concentrations during kisspeptin-10 infusion although serum testosterone increased significantly. Androgens have been shown to down -regulate *Kiss1* gene expression in the non-human primate hypothalamus (Shibata, Friedman et al. 2007) and kisspeptin-secreting neurones have been proposed to constitute part of the pathway mediating sex steroid negative feedback (Oakley, Clifton et al. 2009). Despite significant increases in serum testosterone, although within the normal range, there was no late decline in LH suggesting that within the physiological range, testosterone has limited ability to modulate the ability of the GnRH neuron to respond to kisspeptin-10 and the pituitary gonadotropes to respond to GnRH.

It has to be noted that the responses to 3-μg/kg bolus of kisspeptin-10, administered at the start of the infusion was comparable to the responses observed in studies described in chapter 1. In chapter 1, comparison is made between vehicle and various doses of kisspeptin-10 studies analysing variance simultaneously across various groups. In the present experiment however, comparison is made between LH concentrations before and after the 3 μg/kg dose, giving rise to apparent inconsistency. However, as outlined in section 4.4.1.1, all doses of kisspeptin-10 studied in experiments described in chapter 1 elicited a significant rise in LH from baseline. This indicates that kisspeptin responsiveness to a given dose is consistent. The translational applications of this are described in chapter 8.

While three subjects demonstrated an increase in LH pulse frequency in response to the higher-dose kisspeptin-10 infusion, one did not. Deconvolution analysis (Liu, Keenan et al. 2009), like other algorithms to detect LH pulses (see section 1.1.3.5) is less sensitive with high LH secretion rates as the distinct increase in LH concentration achieved by individual pulsatile discharges of LH can be obscured. The high mean LH concentrations during the higher dose kisspeptin-10 infusion are likely to have prevented reliable analysis of pulse frequency leading to underestimation.

Stimulatory effects of kisspeptin-10 on LH, FSH and testosterone established in this study could inform future studies using kisspeptin as a diagnostic or therapeutic agent. Potent kisspeptin agonists (Curtis, Cooke et al. 2009) and antagonists (Millar, Roseweir et al. 2010) currently being tested in animal models are amino acid substitutions of the decapeptide sequence of kisspeptin-10. Moreover, we have also demonstrated that GnRH responsiveness is preserved in subjects receiving kisspeptin-10 infusion and that maximal LH responses seen with kisspeptin-10 are considerably lower than that achieved with GnRH. Therefore, kisspeptin-10 might provide a more physiological stimulation of the human reproductive axis with potential therapeutic advantages in stimulation of ovulation.

Our study protocols did not incorporate measurement of kisspeptin immunoreactivity for two reasons. With the expected very short plasma half-life of kisspeptin-10 (12), the post-injection blood sampling schedule would need to include samples much earlier and more frequently than just at 10 minutes, for accurate analysis. Moreover, immunological assays can potentially give ‘false-positive’ results in the presence of cleavage products of the peptide as non-specific binding of kisspeptin antibodies is well established (42). Mass-spectroscopic assays of serum samples for kisspeptin 10 at various time-points during the 4 μg/kg/hr kisspeptin-10 infusion were therefore attempted by collaborators (Pfizer, Sandwich, Kent, UK) but concentrations of kisspeptin-10 and its cleavage products (kisspeptin 9, 7 or 4) were below the detection limit (1 ng/ml) at almost all time points, most likely due to suboptimal sample processing.

This study demonstrates the novel phenomenon that continuous infusion of kisspeptin-10 increases LH pulse frequency. Along with increased secretory mass in individual pulses, this increase in pulse frequency resulted in pulsatile secretion contributing to a higher proportion of total LH secreted. Data from other investigators has not shown that GnRH pulse frequency is increased by an acute bolus of kisspeptin-10 (Chan, Butler et al. 2011), indicating the need for continuing kisspeptin stimulation, to study it's influence on GnRH pulse frequency. The dependency of LH pulsatility on pulsatile GnRH secretion (Clarke and Cummins 1985; Millar, Lu et al. 2004) is well established, and kisspeptin has been shown to directly stimulate GnRH secretion (Messager, Chatzidaki et al. 2005) with electrophysiological studies showing intense and prolonged activation of rodent GnRH neurons by kisspeptin-10 (Han, Gottsch et al. 2005). There is a marked stochastic increase in LH when kisspeptin is applied to the hypothalamic arcuate nucleus in rodents which might reflect an acceleration of LH pulse frequency (Li, Kinsey-Jones et al. 2009) and a kisspeptin antagonist decreases LH pulse frequency in this model (Li, Kinsey-Jones et al. 2009). Systemic administration of kisspeptin-10 in our study prevents us from concluding whether this increase in LH (and by inference GnRH) pulse frequency is mediated by a direct effect on GnRH neurons (Krsmanovic, Hu et al. 2009) or through stimulation of the hypothalamic pulse generator (Ohkura, Takase et al. 2009). However, we have demonstrated that LH pulsatility is dependent on kisspeptin, increasing significantly during continuous administration. However, it is possible that endogenous release of kisspeptin is pulsatile, and pulsatile administration may have a different impact on GnRH pulsatility, modulated by co-transmitters such as neurokinin B and dynorphin (Wakabayashi, Nakada et al. 2010). There may also be species and sex differences in GnRH pulse regulation as unlike rodents, a GnRH surge is not obligatory for the preovulatory LH surge in women (Adams, Taylor et al. 1994).

An increase in LH pulse size (secretory mass per pulse or burst size) was observed in our study, with pulse sizes computed by a well validated, automated analytical program. This increase in secretory mass in individual pulses observed during kisspeptin-10 infusion can be a function of two factors either in isolation or in

combination – one, an increase in the quantity of GnRH secreted and two, an increase in pituitary sensitivity to GnRH. As no known changes in the hormonal milieu with a potential to increase gonadotrope sensitivity arises from kisspeptin administration, it is logical to conclude that kisspeptin augments GnRH pulse size. However, one has to be cognisant that the deconvolution algorithm employed in the present study – as well as other algorithms to assess LH pulsatility as discussed in section 1.1.3.5 – is designed to assess physiological LH secretion, where adjacent pulses are sufficiently demarcated to allow a clear definition of baseline. When a subsequent pulse is generated before LH concentration return to nadir, the identification of 'baseline' needs mathematical modelling and may not be reflective of underlying physiology. Interestingly, pioneering investigators of LH pulsatility had identified this difficulty in differentiating 'basal' and 'pulsatile' secretion (Yen, Tsai et al. 1972). Three decades on, these challenges remain –and are likely to remain for the foreseeable future. With a short half-life (12-15 min) and good correlation with LH, free α subunit (FAS) could be used a surrogate of GnRH pulse frequency. Although there is desensitisation in free α subunit secretion at rapid GnRH frequencies, it has been shown to be a superior marker to LH at GnRH frequencies around 30 min but the sensitivity of FAS and LH are comparable at the pulse frequencies observed in the present study in the region of a pulse every hour (Hayes, McNicholl et al. 1999).

In conclusion, continuous infusion of kisspeptin-10 increases mean serum LH, LH pulse frequency, LH secretory mass-per-burst and testosterone secretion without rapid desensitisation/tachyphylaxis of effect observed in other species.

6 Gonadotropin responses to kisspeptin-10 in patients with pubertal delay

6.1 Introduction

Reactivation of the GnRH pulse generator that is active in infancy but quiescent in childhood with associated increase in LH pulse frequency is critical for pubertal development (Roth, Kelch et al. 1972; Conte, Grumbach et al. 1980; Waldhauser, Weissenbacher et al. 1981; Balasubramanian, Dwyer et al. 2010). As discussed in section 1.4, precocious puberty is associated with premature activation of GnRH pulse frequency and pubertal delay associated with delayed activation of the same.

Having established that kisspeptin-10 evokes robust LH responses (Chapter 3) and that kisspeptin-10 infusion enhances LH pulse frequency in healthy men (Chapter 5), we sought to establish the kisspeptin-10 response profile in patients with GnRH deficient phenotype. Towards this, kisspeptin-10 responses were assessed in patients with pubertal delay associated with neurokinin B (NKB) signalling deficiency (see chapter 1.3.4)

6.2 Objectives and hypothesis

- In patients with NKB signalling defects, we hypothesised that kisspeptin-10 will evoke LH secretion, demonstrating that NKB signalling is not obligatory for kisspeptinergic stimulation of GnRH neurons.

Studies reported herein are observational studies in small number of patients with a clear reproductive phenotype and provides preliminary data. Studies of larger cohorts of such patients are required to fully test the hypotheses.

6.3 Studies in patients with neurokinin B signalling defects

6.3.1 Participants

Two patients each, with loss of function mutations in the NKB gene (*TAC3*) (13) and the gene encoding NK3R (*TAC3R*) were recruited from Bicêtre Hospital (Paris, France). Patients harbouring *TAC3* mutations were both men (aged 21 and 31), while a 26 year-old woman and 28 year old man harbouring *TAC3R* mutations also took

part. The Paris Sud University and Bicêtre Hospital ethics committees approved the study and all participants gave their informed consent (Dr. Jacques Young).

Patients 1 and 2 harbour the c.209-1G_C homozygous mutation in the NKB gene (*TAC3*)(13). This mutation is located in the IVS3 acceptor splice-site and unmasks a cryptic splice-site in IVS3, leading to the insertion of 22 nucleotides between exons 3 and 4 in the transcript. This insertion results in a frame-shift downstream of codon 67 and the emergence of a premature stop codon at position 76, causing the termination of translation upstream of the NKB coding region. Patient 3 has a homozygous deletion (c.483_499del) in the gene encoding NK3R (*TAC3R*). This deletion leads to a frameshift downstream of codon 161 resulting in a premature stop codon at position 183 truncating the NK3R after the third transmembrane domain.

Patient 4 harbours the homozygous c.738-1G_A mutation in the NK3R gene. This results in the loss of the natural splice acceptor site of intron 2 resulting in truncation of the receptor after the second extracellular loop, thus eliminating transmembrane domains 5–7 and the associated intracellular and extracellular loops (Young, Bouligand et al. 2010).

Testosterone and estradiol replacement in the male and female patients were discontinued three months prior to study visits.

6.3.1 Protocol

This study was undertaken in collaboration with Dr. Jacques Young at the Bicêtre Hospital (Paris, France). Volunteers were admitted to the Bicêtre Hospital at 0800 for 12 hours of blood sampling every 10 minutes for 2 consecutive days. On the first day, normal saline was infused as vehicle and on the second, kisspeptin-10 was administered at a rate of 1.5 μg/kg/hr for 11 hrs based on observations in healthy volunteers reported in chapter 5. LH, FSH, testosterone, estradiol and inhibin B levels were measured in Paris (Dr. Jacques Young) using methods previously reported (13). LH and FSH pulses were identified using the Thomas algorithm (an adaptation of the Santen –Bardin method of pulse detection)(Young, Bouligand et al. 2010). Additionally, serial LH data was also analysed using the deconvolution analysis described in chapter 3 to determine the number of pulses, LH pulse size (burst mass) and the relative contribution of basal and pulsatile secretion of LH to

total LH secretory output (Dr. Johannes Veldhuis). These two analytical methods were chosen as there is no 'gold standard' method of pulse detection and different pulse detection algorithms have different sensitivity and specificity as summarised in section 1.1.3.

6.3.2 Results

6.3.2.1 Mean LH, FSH and Inhibin B

Administration of kisspeptin-10 increased mean LH, FSH and Inhibin B concentrations as summarized in the table 6.1 below. Serial gonadotropin data during baseline LH pulsatility analysis and kisspeptin-10 infusion in the four subjects studied is summarized in figure 6.1.

	LH (IU/L)		FSH (IU/L)		Inhibin B (pg/mL)	
	Saline	Kisspeptin	Saline	Kisspeptin	Saline	Kisspeptin
P1/M	0.2±0.1	0.4±0.2***	15.9±3.2	27.4±4.6*	2.9±0.2	3.4±0.5***
P2/M	0.62±0.41	1.21±0.60***	10.4±1.6	19.1±3.1*	4.4±0.6	7.1±1.1***
P3/F	0.22±0.06	1.25±0.56***	8.15±2.9	26.2±4.7**	2.05±0.1	4.2±0.9***
P4/M	0.42±0.10	1.12±0.43***	66±7.4	112±13.7**	3.0±0.2	5.4±0.7***

Table 6.1: LH, FSH and Inhibin B Responses to kisspeptin-10 and normal saline in patients with mutations in *TAC3* (patients 1 and 2) or *TACR3* (patients 3 and 4). *p< 0.05 ; **p<0.01; ***p<0.0001 (paired t test) ; M : male, F :female. At both visits, 12 random measurements of inhibin B were performed.

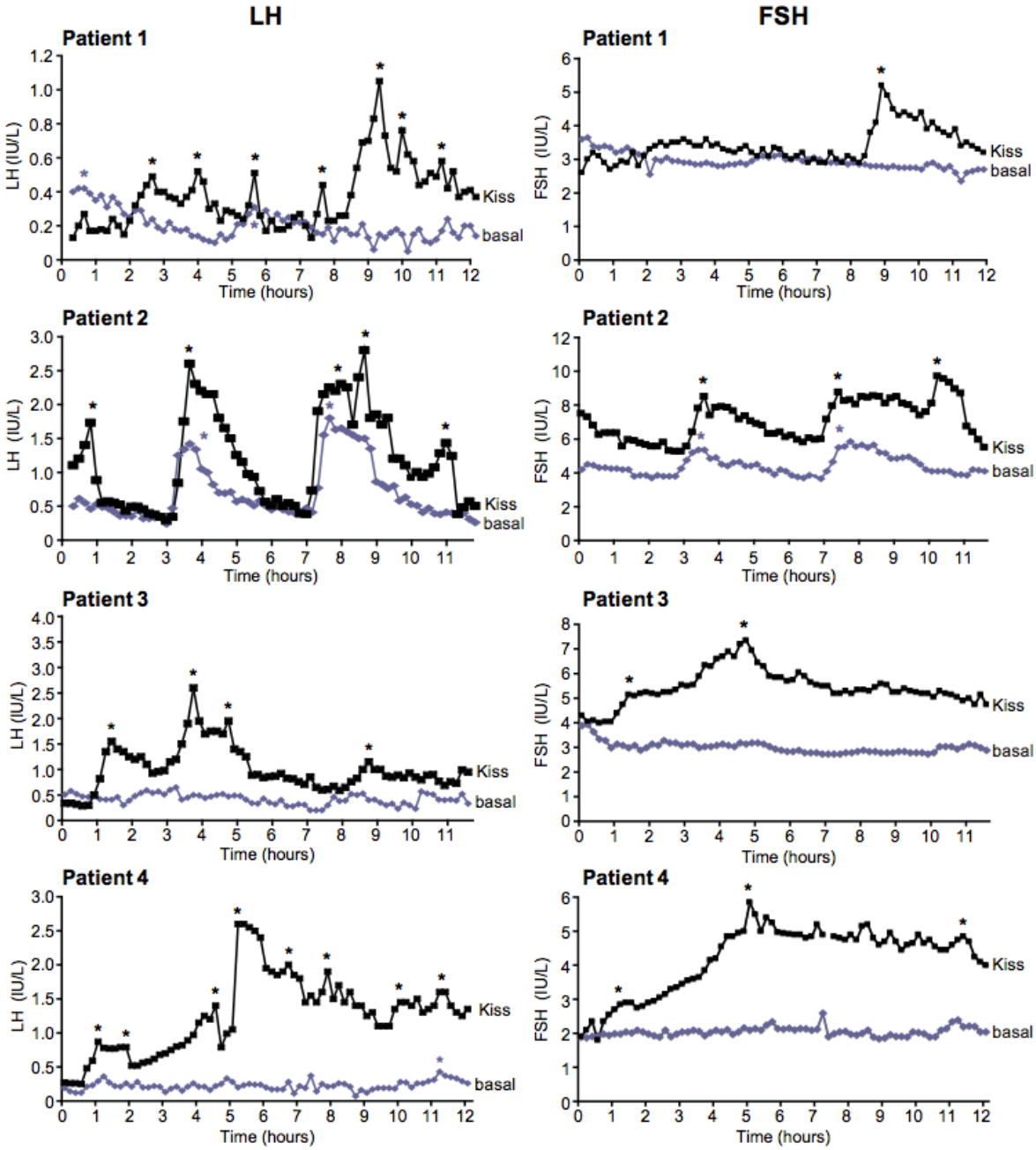


Figure 6.1 Serum LH and FSH concentrations in patients with neurokinin B signalling defect. Blue lines denote basal LH and FSH profiles and black lines are used to represent values observed during kisspeptin-10 infusion. Asterisks represent LH and FSH pulses detected using the Thomas algorithm (an adaptation of the Santen –Bardin method of pulse detection).

6.3.2.2 Mean Testosterone and Estradiol

Increases in gonadotropin concentrations were associated with significant increases in sex steroids in all the four subjects studied (Figure 6.2).

	Testosterone (ng/mL)		Estradiol (pg/mL)	
	Saline	Kisspeptin	Saline	Kisspeptin
P1/M	0.13±0.1	0.4±0.2*	-	-
P2/M	0.12±0.08	0.6±0.14*	-	-
P3/F	-	-	6.4±1.9	18.2±3.1**
P4/M	0.42±0.10	1.12±0.43*	-	-

Table 6.2: Gonadal steroid responses to kisspeptin-10 and vehicle in patients with *TAC3* (patients 1 and 2) or *TACR3* (patients 3 and 4) mutations. *p< 0.05; **p<0.01; ***p<0.0001 (paired t test); M : male, F :female. - : Not determined.

6.3.2.3 LH pulse frequency

LH and FSH pulse frequency increased significantly during kisspeptin-10 infusion compared to vehicle (Figure 6.2) when data was analysed using Thomas algorithm.

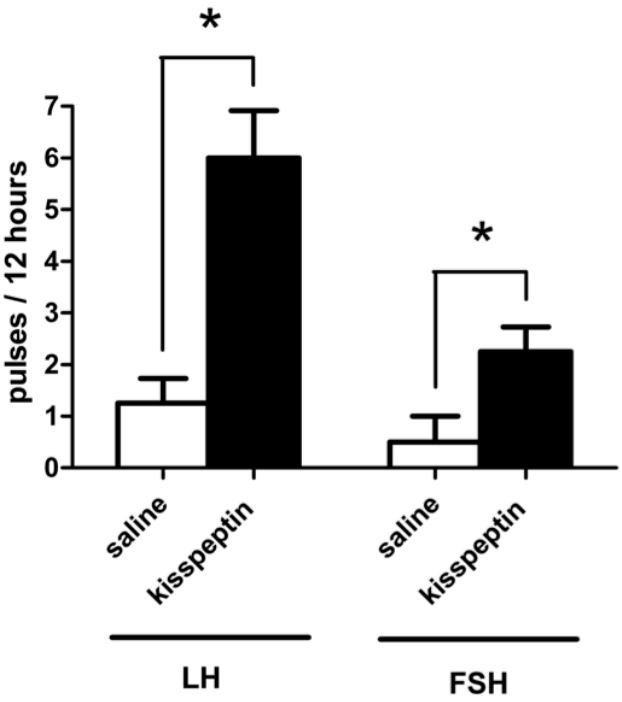


Figure 6.2 Changes in LH and FSH pulse frequency in patients with neurokinin B signalling defect. * P<0.05 (paired t test).

Deconvolution analysis demonstrated an increase in LH pulse frequency in the two patients with TAC3 mutations from seven to ten, and from five to eight pulses respectively in 12 hours. However, in patients with *TACR3* mutations, LH pulse frequencies during saline and kisspeptin-10 infusions were unchanged (6 and 7 pulses every 12 hours).

Series	Number of pulses	Basal LH	Pulse LH	Total LH	MPP
P1 Basal	7.0	2.3	1.6	4.0	0.2
P1 Kp	10.0	4.5	4.4	8.9	0.4
P2 basal	5.0	7.3	9.6	16.9	1.9
P2 Kp	8.0	4.8	23.1	27.9	2.9
P3 basal	6.0	5.4	5.2	10.6	0.9
P3 Kp	6.0	15.8	9.8	25.5	1.6
P4 basal	7.0	3.4	2.8	6.1	0.4
P4 Kp	7.0	10.5	4.9	15.4	0.7

Table 6.3: Deconvolution analysis of LH pulse frequency. Subjects 1 and 2, with neurokinin B secretory defects showed an increase in LH pulse frequency while subjects 3 and 4 (with TACR3 mutations) show similar pulse frequency at baseline and during kisspeptin-10 infusion

6.4 Discussion

The present exploratory study, although carried out in a small number of subjects, provides some insights into the neuroendocrine regulation of human reproduction.

As discussed in section 1.2.3, evidence from animal models and *in vitro* studies suggest that kisspeptin stimulation of gonadotropin secretion is mediated through the GnRH neuron. The observation that kisspeptin-10 has no effect on LH secretion in men with Kallmann Syndrome (with functional impairment of GnRH neurons) is consistent with this notion. However, it has to be noted that these men were on exogenous testosterone therapy and their pituitary gonadotropes were not primed with prior exposure to GnRH.

Kisspeptin-10 infusions effected a small but significant increase in both LH and FSH secretion in patients with neurokinin B signalling defects caused by TAC/TACR3

mutations. These observations suggest that neurokinin B signalling not obligatory for kisspeptinergic stimulation of gonadotropin secretion and are consistent with studies in animal models. In primates, neurokinin B antagonist abrogated neurokinin B dependant stimulation LH secretion whilst having no impact on kisspeptinergic stimulation of LH (Ramaswamy, Seminara et al. 2010).

Discordance of results between LH pulse frequency using the Thomas algorithm and a deconvolution algorithm highlights the challenges posed by the lack of a universally valid 'gold-standard' method to perform gonadotropin pulsatility analysis (described in section 1.1.3.5). However, it has to be noted that the two patients with TAC3 signalling showed an increase in LH pulse frequency on deconvolution analysis whilst patients with TACR3 receptor did not. Further studies in larger number of participants are required to explore whether these observations can be reproduced in larger cohorts of patients.

Whilst LH and FSH secretion were stimulated during kisspeptin-10 infusion the resultant mean concentrations and pulse frequency were lower than that observed in healthy volunteers (chapter 5). There are two potential explanations. One, that the pituitary gonadotropes in these patients were not been primed by normal GnRH pulsatility. Secondly, it is possible that neurokinin B has an additive positive impact on GnRH output that is, at least in part, independent of kisspeptin signalling. Development of specific kisspeptin antagonists that are effective on peripheral administration in humans will be an important step in exploring hierarchical regulation of reproductive function in the human hypothalamus.

7 Translational application of kisspeptin-10 physiology: Normalisation of testosterone secretion in hypogonadal men with type 2 diabetes.

7.1 Introduction

Low serum testosterone concentration is observed in one-quarter to one-half of men with type 2 diabetes (Dhindsa, Prabhakar et al. 2004; Kapoor, Aldred et al. 2007; Grossmann, Thomas et al. 2008; Dandona and Dhindsa 2011). Men with type 2 diabetes have lower free testosterone concentrations when compared to age- and BMI-matched men (Dhindsa, Miller et al. 2010) and the prevalence of hypogonadism in this population increases with age and BMI (Ding, Song et al. 2006; Grossmann, Thomas et al. 2008). Low endogenous testosterone is associated with significant long-term health implications and is associated with increased risk of all-cause mortality and cardiovascular death in men (Araujo, Dixon et al. 2011). Decreased serum testosterone in the vast majority of men with type 2 diabetes is not accompanied by a compensatory increase in pituitary secretion of luteinising hormone (LH) (Dhindsa, Prabhakar et al. 2004; Dandona and Dhindsa 2011), suggesting that hypothalamic-pituitary dysfunction is part of the pathophysiology of hypogonadism associated with type 2 diabetes (Hill, Elmquist et al. 2008; George, Millar et al. 2010). However, the neuroendocrine mechanisms underlying this relative deficit in LH secretion remain to be elucidated (Hill, Elmquist et al. 2008).

The central role for GnRH neurons as the final integrator of metabolic and endocrine modulators of the hypothalamic-pituitary-gonadal axis is well established (Millar, Lu et al. 2004). Pituitary secretion of LH, and thereby testicular testosterone production, is regulated by changes in the amplitude and frequency of pulsatile hypothalamic GnRH secretion (Millar, Lu et al. 2004; Balasubramanian, Dwyer et al. 2010). Novel neuropeptide regulators of GnRH have recently been identified, with kisspeptins being crucial for pulsatile GnRH secretion, as demonstrated in both human and animal studies (de Roux, Genin et al. 2003; Seminara, Messager et al. 2003; Roseweir and Millar 2009). Studies in experimental animals have shown changes in *Kiss1* gene expression in response to factors known to lower GnRH secretion,

including hyperglycaemia (Castellano, Navarro et al. 2006), inflammation (Iwasa 2008), and estrogen feedback (Oakley, Clifton et al. 2009), suggesting that decreased endogenous kisspeptin secretion may account for decreased hypothalamic-pituitary-gonadal function in men with type 2 diabetes (George, Millar et al. 2010).

Low testosterone in men with type 2 diabetes is associated with an adverse cardio-metabolic phenotype – decreased high density lipoproteins, increased triglycerides, increased body mass index and insulin resistance (Grossmann, Thomas et al. 2008). Moreover, in men with type 2 diabetes, decreased testosterone concentrations are associated with a three-fold increase in cardiovascular mortality (Ponikowska, Jankowska et al. 2010). While there is a paucity of long-term clinical outcomes in relation to testosterone replacement therapy in these men (Bhasin, Cunningham et al. 2010), recent short-term studies suggest improvements in cardiovascular-risk profile following testosterone replacement in hypogonadal men with type 2 diabetes (Jones, Arver et al. 2011). However, in individuals at high-risk for cardiovascular disease, testosterone therapy (where serum testosterone concentrations often exceed the physiological range) has been associated with serious adverse events (Basaria, Coviello et al. 2010). Stimulation of endogenous testosterone secretion by enhancing GnRH secretion and increasing testosterone within the physiological range (chapter 3), may therefore be therapeutically advantageous in this context.

7.2 Objectives and hypothesis

Clinical studies were designed to test the hypothesis that kisspeptin mediates hypogonadotropic hypogonadism in men with type 2 diabetes and that administration of exogenous kisspeptin will stimulate LH and testosterone secretion (George, Millar et al. 2010). Acute responses in serum LH to maximally stimulatory intravenous doses of kisspeptin-10 and GnRH in hypogonadal men with type 2 diabetes were compared with those in healthy volunteers. Kisspeptin-10 was subsequently administered intravenously for 11 hours to assess the effects of sustained stimulation on LH and testosterone secretion.

7.3 Research Design and Methods

7.3.1 Participants

Acute LH responses to kisspeptin-10 and GnRH were assessed in five men with type 2 diabetes and newly diagnosed hypogonadotropic hypogonadism (age 33.6±3 years, BMI 40.6±6.3 kg/m^2, testosterone 7.4±0.7 nmol/L, LH 4.7±0.7 IU/L, FSH 3.4±0.6 IU/L, HbA1c <8 %, duration of diabetes <5 years) and seven age matched healthy men (age 31.2±1.8 years, BMI 25±1.6 kg/m^2, LH 5.5±0.8 IU/L, FSH 4±0.6 IU/L, testosterone 19.6±1.5 nmol/L, normal glucose tolerance). Four men with type 2 diabetes also participated in the kisspeptin-10 intravenous infusion study.

Studies were approved by the Lothian Research Ethics Committee (Ref 09/S1101/23) and all participants provided written informed consent. All volunteers had normal secondary sexual characteristics with minimum total testicular volume of 40 ml and normal physical examination. Baseline full blood count, renal function, liver function and electrolytes were within normal limits. Healthy volunteers underwent a 75-g glucose tolerance test to demonstrate normoglycaemia.

7.3.2 Study design

7.3.2.1 Acute kisspeptin-10 and GnRH stimulation study.

This study was designed to compare acute LH responses to kisspeptin-10 in hypogonadal men with type 2 diabetes with age-matched healthy volunteers and involved two 6-hr visits to the Edinburgh Clinical Research Facilities, at least a week apart. All visits commenced between 0800 and 0900 following overnight fast to avoid diurnal and nutritional bias. Blood samples were obtained at 15-min intervals for three hours following which an intravenous bolus of 100 μg of GnRH was administered at the first visit and 0.3 μg/kg (0.23 nmol/kg) kisspeptin-10 at the subsequent visit. Samples were collected at 15-min intervals for a further three hours. Standardised meals were provided. Blood pressure, heart rate and peripheral oxygenation were monitored regularly throughout the study. Full blood count, serum electrolytes, liver function, renal function and serum glucose were measured at the start and the end of all visits as safety measures as kisspeptins had not previously been administered to men with diabetes.

7.3.2.2 Kisspeptin-10 infusion study

This study was designed to assess whether the persistent and robust increase in mean serum LH concentration, LH pulse frequency, LH secretory-burst per pulse and serum testosterone concentration during continous infusion of kisspeptin-10 previously demonstrated in healthy volunteers (Chapter 3) was reproducible in hypogonadal men with type 2 diabetes.

Volunteers attended for two 12-hr visits, five days apart. Both visits involved collection of 2 mls of blood at 10-min interval for 12 hours. At the second visit, baseline sampling was performed for one hour following which kisspeptin-10 was infused intravenously (Crono PCA, Cane, Rivoli, Italy) at 4 μg/kg/hr. Standardised meals were provided and safety checks carried out as in the acute kisspeptin-10 stimulation study.

7.3.3 Study Drugs

Kisspeptin-10 was custom-synthesised to GMP standards (Bachem GmBH, Weil am Rhein, Germany) with 97% purity on HPLC with a mass balance of 98.8% and prepared by reconstituting 1 mg kisspeptin-10 in 5 ml normal saline (Chapter 2). Maximally stimulatory dose of kisspeptin-10 (0.3 μg/kg) was identified from our dose-escalation study in healthy male volunteers (Chapter 3). The infusion rate of 4 μg/kg/hr was chosen to achieve maximal stimulation of LH secretion with a view to having maximal stimulation of endogenous testosterone secretion. Although a lower dose (1.5 μg/kg/hr) was shown to enhance LH pulse frequency in healthy men (Chapter 5), sustained and robust increase in LH secretion was observed without any evidence of desensitisation with the larger 4 μg/kg/hr dose. Commercially supplied GnRH (Relefact, Sanofi Aventis, Frankfurt, Germany) was used for GnRH stimulation.

7.3.4 Hormone assays

Blood-samples were batch-processed by centrifugation at 4^{o}C for 10 min at 3000 rpm and serum was frozen at -20^{o}C until analysis. LH was assayed in all samples by ELISA and testosterone in hourly samples by extraction RIA as previously described (Chapter 2). Samples from individual study visits were analysed together. Inter-assay

coefficients of variation for all hormonal assays were <5 % at the concentrations measured. Intra-assay coefficient of variation of LH was 2.9%.

7.3.5 Statistical Analysis

Data are presented as mean±sem. The statistical software package Minitab 16 (Minitab Ltd, Coventry, UK) was used. Data not normally distributed were log-transformed prior to analysis. A two-sided P <0.05 was regarded as statistically significant. Sample size calculations were made on the assumption that effect sizes and variance of gonadotropin responses to kisspeptin-10 in hypogonadal men with type 2 diabetes would be comparable to those observed in healthy volunteers (chapter 3).

7.3.5.1 Acute kisspeptin and GnRH stimulation study

As LH secretion is pulsatile, baseline LH for each study visit was calculated as the mean of LH concentrations in the 18 samples during the 3-hr sampling period prior to kisspeptin-10 or GnRH administration. Δ LH was calculated by subtracting this baseline LH value from the peak LH concentration observed following kisspeptin-10 or GnRH stimulation. ΔLH responses to kisspeptin-10 and GnRH in hypogonadal men were compared with healthy controls using two sample Student's *t* tests.

7.3.5.2 Kisspeptin infusion study

LH, FSH and testosterone responses to kisspeptin-10 were calculated by comparing the baseline visit with corresponding data points during kisspeptin-10 infusion using Student's paired *t* test.

A deconvolution algorithm with 93% sensitivity and specificity (Liu, Keenan et al. 2009) was used to calculate basal, pulsatile and total LH secretion on blinded data. This algorithm also provided read-outs of basal (non-pulsatile) and pulsatile components of total LH secretion. Student's paired *t* test was used to compare pulse size and frequency at baseline and during kisspeptin-10 infusion.

Leydig cell sensitivity, i.e. the ratio of serum testosterone to LH concentrations, was calculated during baseline and kisspeptin-10 infusion visits and compared using paired *t* tests.

Additionally, LH pulsatility and Leydig cell sensitivity to LH observed in the study subjects during the baseline visit and kisspeptin-10 infusion were compared to data from contemporaneous study of healthy age-matched volunteers without diabetes using two-sample *t* tests (chapter 3).

7.4 Results

7.4.1 Acute kisspeptin-10 and GnRH responses are preserved in men with type 2 diabetes

LH increased significantly following kisspeptin-10 administration in both hypogonadal men with type 2 diabetes (4.7±0.7 to 10.7±1.2 IU/L P=0.02) and healthy men (5.5±0.8 to 14.5±1.8 IU/L, P =0.002), with peak serum LH observed 30 minutes after kisspeptin in both groups. ΔLH was comparable (6.0±1.3 in men with diabetes vs. 8.9±1.1 IU/L P=0.18, Figure 7.1), with peak LH showing respectively 178±19% and 163±55% increases from baseline.

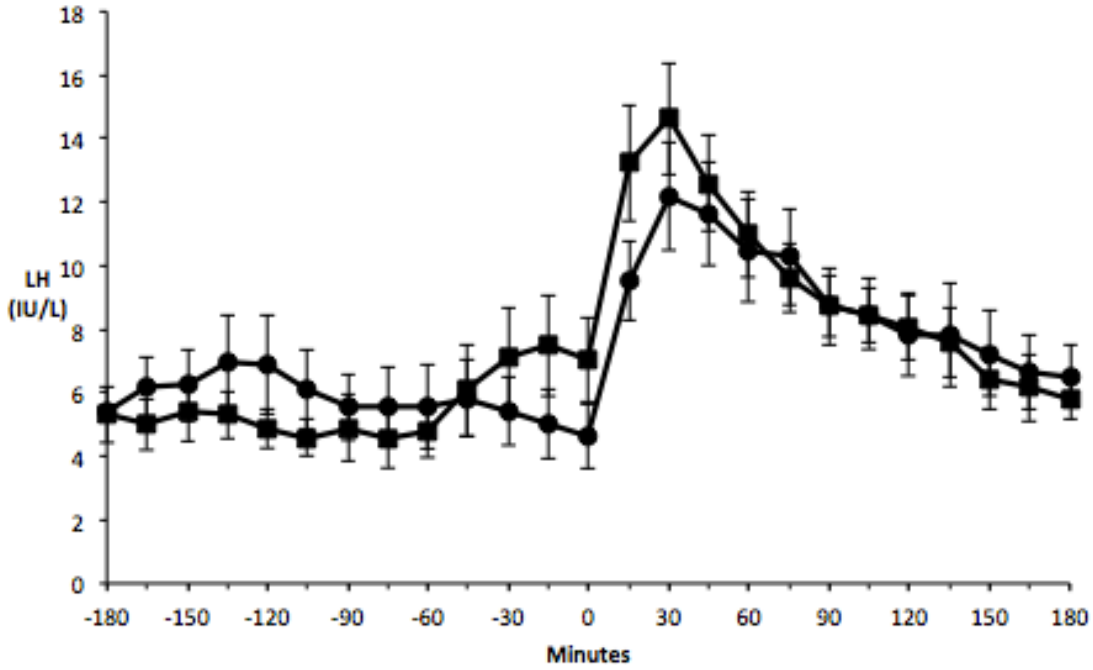


Figure 7.1 Serum LH responses to intravenous bolus of 0.3 µg/kg of kisspeptin-10 in healthy volunteers (squares) and hypogonadal men with type 2 DM (circles).

Following GnRH administration, LH increased from 4.3±0.4 to 37.7±9.9 IU/L in men with hypogonadism and diabetes, and from 5.6±0.6 to 42.0±4.1 in healthy controls. ΔLH response to GnRH was similar in the two groups (ΔLH 36.4±3.9 vs. 33.4±9.9, P=0.41, Figure 7.2).

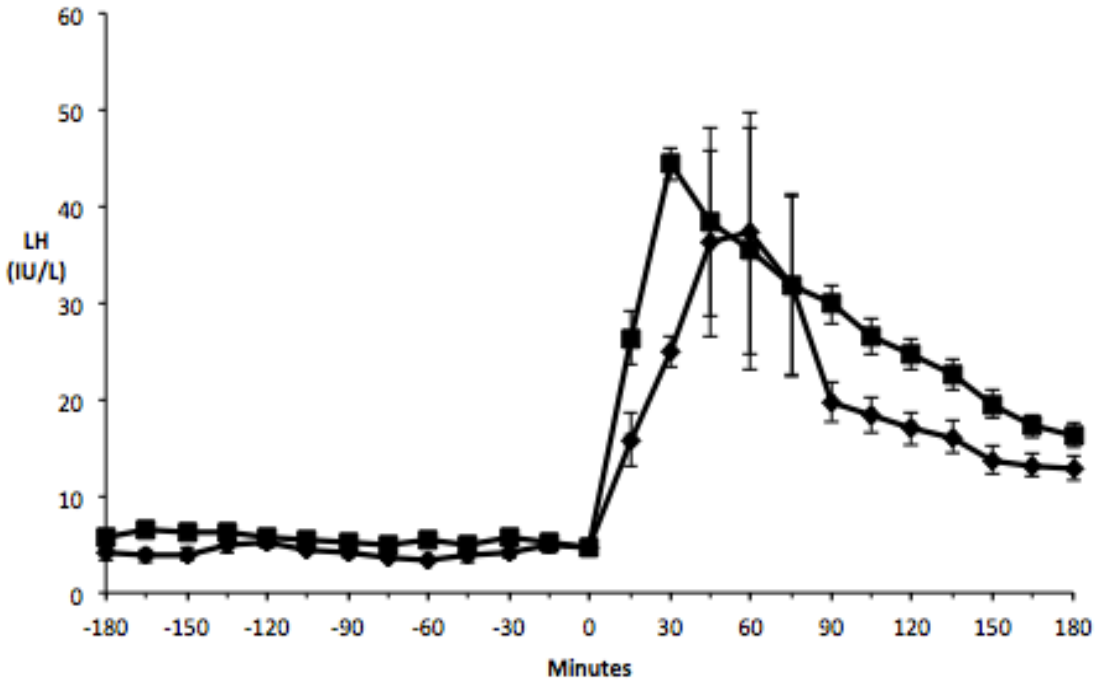


Figure 7.2 Serum LH responses to intravenous bolus of 100 µg GnRH in healthy volunteers (squares) and hypogonadal men with type 2 DM (circles).

7.4.2 Kisspeptin-10 infusion stimulates LH and testosterone secretion in hypogonadal men with type 2 diabetes

LH pulse frequency at baseline was significantly lower in hypogonadal men with diabetes than healthy controls (0.6±0.1 vs. 0.8±0.1 pulses/hr, P=0.03). LH increased significantly during kisspeptin-10 administration in all hypogonadal men with type 2 diabetes. Mean LH increased from 3.9±0.1 at baseline to 20.7±1.1 IU/L during

kisspeptin-10 infusion (P=0.03, Figure 7.3 A-D). LH showed a progressive increase during the first six hours of infusion, tending to plateau thereafter.

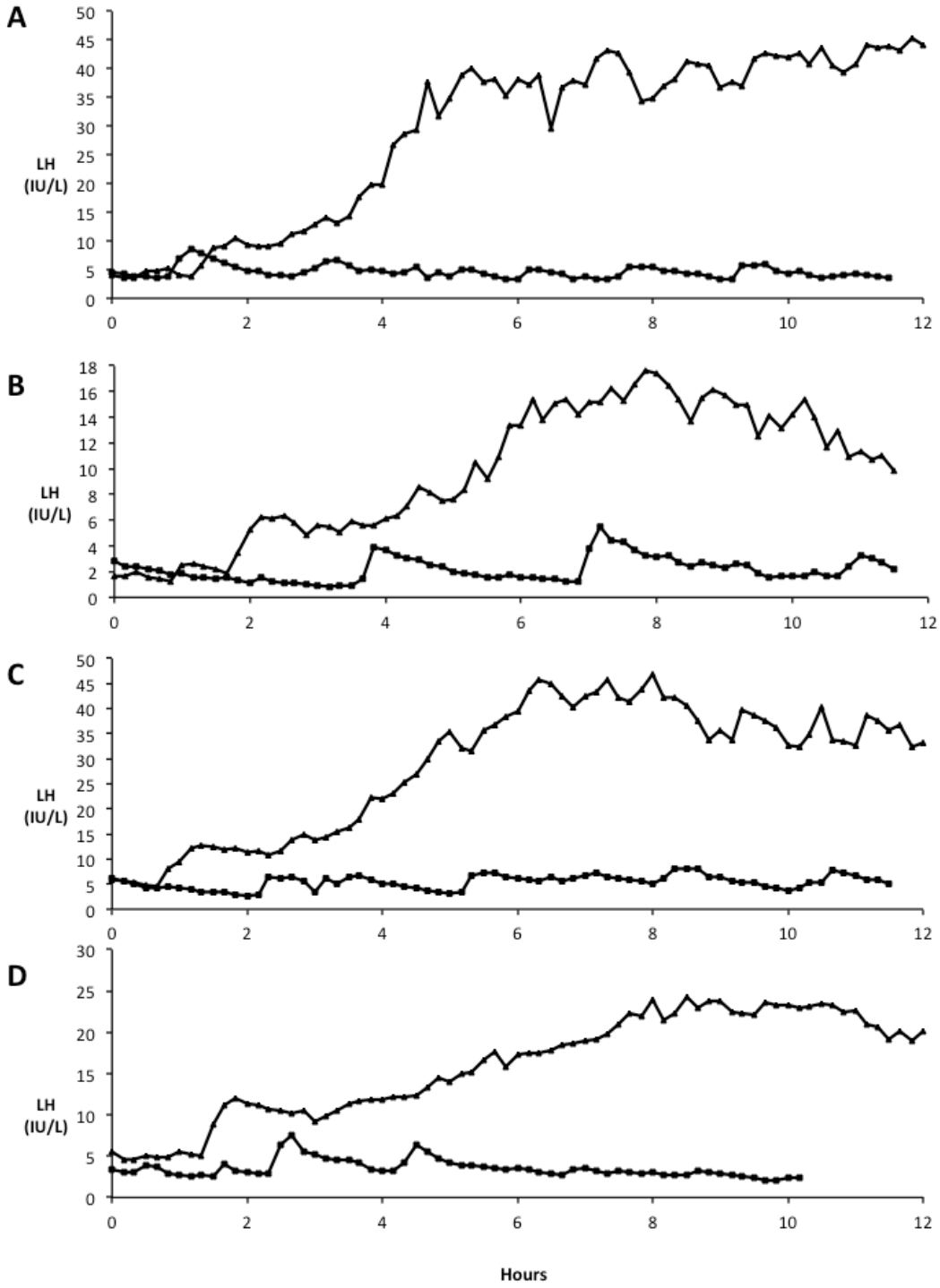


Figure 7.3. Serum LH profile at baseline (squares) and during kisspeptin-10 infusion (triangles) in hypogonadal men with type 2 diabetes. Each of the panels represents a study subject.

The pulsatile component of LH secretion, calculated using deconvolution of LH data obtained at 10-min intervals, showed a significant increase from 32.1±8.0 IU/L at baseline to 140.2±23.0 during kisspeptin-10 infusion (P=0.007). Mean pulse frequency increased from 0.6±0.1 pulses/hr at baseline to 0.9±0 pulses/hr during kisspeptin-10 infusion, (P=0.05 Figure 7.4A) and LH secretory mass per pulse increased from 5.1±0.6 to 15.2±2.6 IU/L (P=0.017 Figure 7.4B). Pulse frequency during kisspeptin-10 infusion was also comparable to data from age-matched healthy male volunteers (0.9±0 vs. 1.0±0.1 pulses/hr in healthy controls P=0.4).

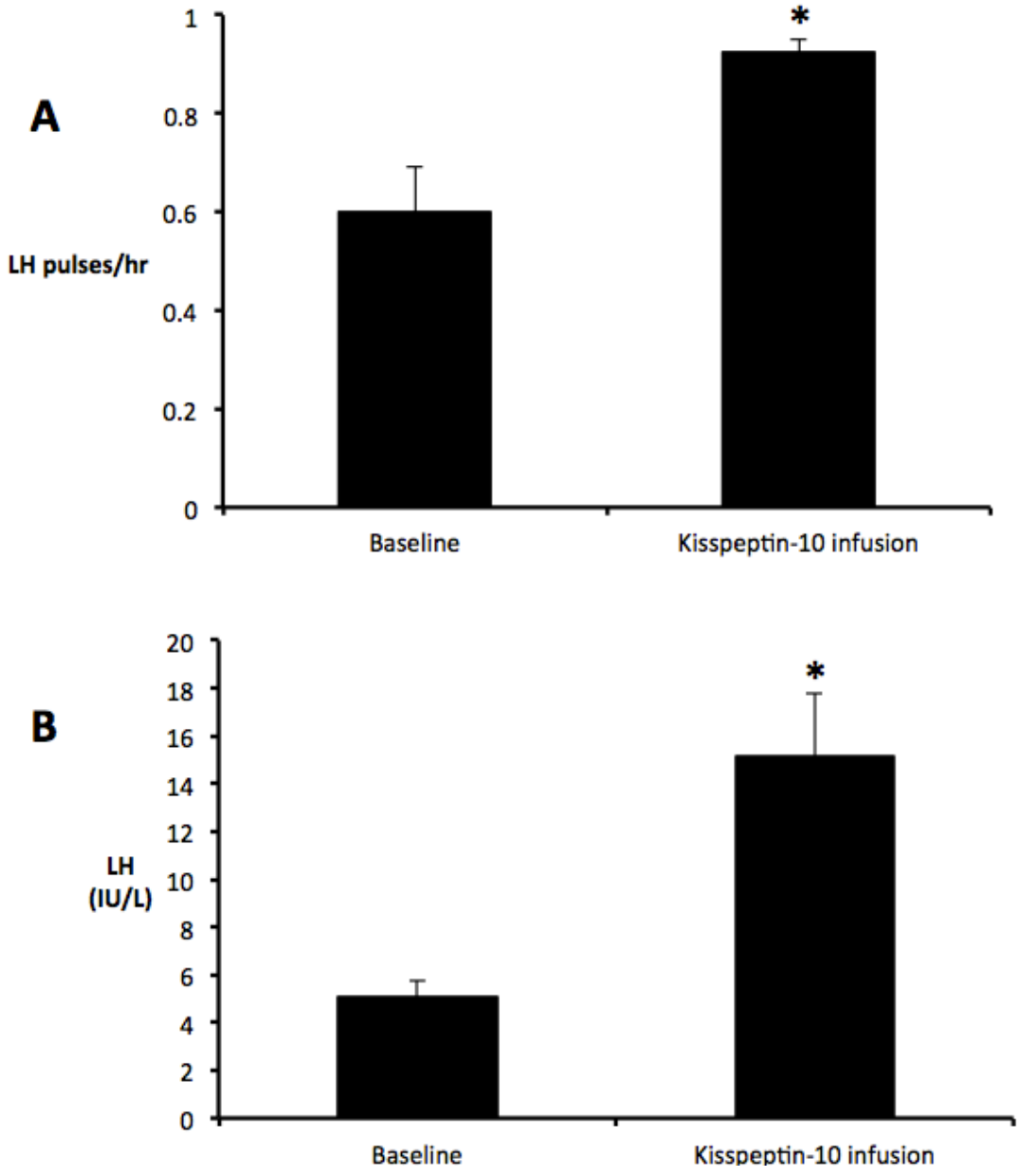


Figure 7.4 LH responses to kisspeptin-10 infusion in hypogonadal men with type 2 diabetes. A, LH pulse frequency increased from 0.6±0.1 to 0.9±0 pulses/hr during kisspeptin-10 infusion (P=0.05). B, LH secretory-burst (mass per pulse) increased from 5.1±0.6 to 15.2±2.6 IU/L (P=0.017). Mean testosterone increased from 8.5±1.0 at baseline to 11.4±0.9 nmol/L at the end of kisspeptin-10 infusion, P=0.002, (Figure 7.5). Leydig cell responsiveness in hypogonadal men with T2DM, as estimated as the ratio of serum testosterone to LH, were also comparable with healthy volunteers at both the baseline visit (4.6±1.1 in T2DM vs. 2.7±0.9, P=0.23) and during the kisspeptin-10 infusion visit (1.7±0.4 in T2DM vs. 0.9±0.3, P=0.76).

Serum FSH concentration did not show a significant change during kisspeptin-10 infusion (3.9±1.1 IU/L at baseline vs. 5.5±1.1 IU/L, also consistent with observations in healthy male volunteers (Chapter 3).

No adverse events were identified.

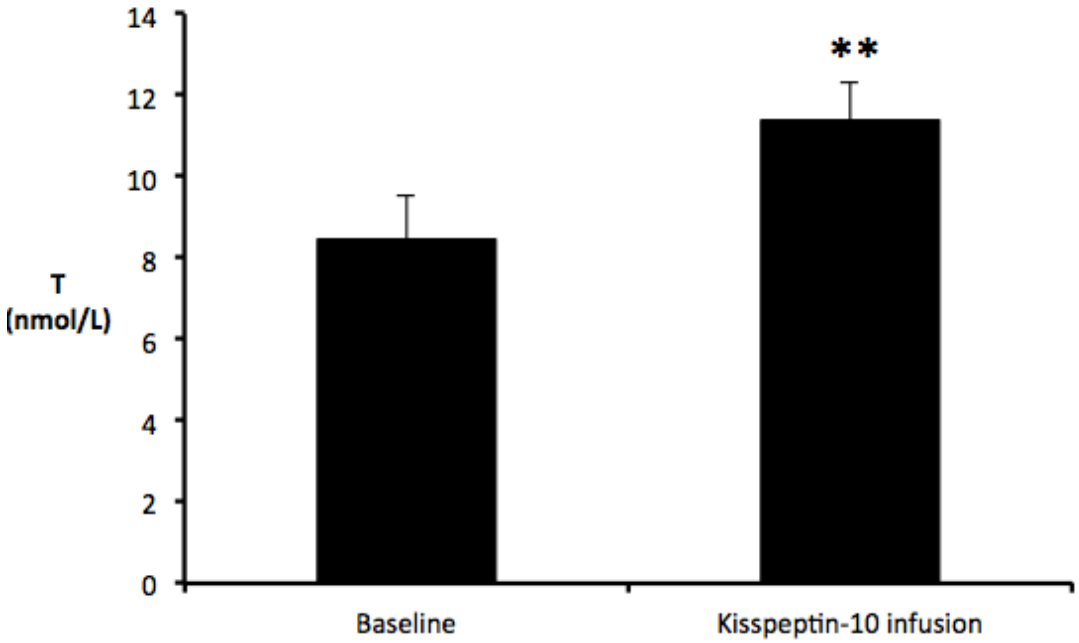


Figure 7.5 Testosterone response to kisspeptin-10 infusion in hypogonadal men with type 2 diabetes. Mean testosterone increased from 8.5±1.0 at baseline to 11.4±0.9 nmol/L at the end of kisspeptin-10 infusion (P=0.002).

7.5 Discussion

Our acute studies in hypogonadal men with diabetes demonstrate normal responsiveness of the hypothalamic GnRH neuronal population to kisspeptin-10, and of pituitary gonadotropes to GnRH. Testosterone concentrations also normalised during kisspeptin-10 infusion in response to increased LH, suggesting that the cause of the hypogonadism is predominantly central in origin, in keeping with the demonstration here that basal LH pulse frequency is low, and observations in rodent models of experimental diabetes (Castellano, Navarro et al. 2006). Normalisation of testosterone during continuous kisspeptin-10 infusion with testosterone to LH ratios comparable to healthy male volunteers suggests that Leydig cell responsiveness to LH is also preserved in hypogonadal men with type 2 diabetes.

Kisspeptin signalling through its cognate receptor GPR54 (*Kiss1R*) has been proposed as one of the mechanisms whereby metabolic signals modulate the hypothalamic-pituitary-gonadal axis (Castellano, Bentsen et al. 2010; George, Millar et al. 2010). Studies in animal models have shown that factors associated with hypogonadism in diabetes such as hyperglycaemia (Castellano, Navarro et al. 2006), inflammation, decreased leptin input (Smith, Acohido et al. 2006) and increased estrogen feedback (Oakley, Clifton et al. 2009) down-regulate *Kiss1* gene expression. Our observations are consistent with the notion that the hypothalamic-pituitary-gonadal axis *per se* is functionally intact in hypogonadal men with type 2 diabetes and that the reduced activity of the hypothalamic-pituitary-gonadal axis occurs in response to perturbed afferent inputs, which include metabolic and endocrine pathways.

A direct pathological effect of diabetes on Leydig cell function has been proposed as some men with type 2 diabetes show submaximal testosterone response to hCG stimulation (Ermetici, Donadio et al. 2009). Insulin resistance has also been demonstrated to be associated with decreased serum testosterone in men (Pitteloud, Hardin et al. 2005). However, our results show robust Leydig cell response to kisspeptin-stimulated endogenous LH comparable to observations in healthy male volunteers suggesting that Leydig cell sensitivity to LH may be preserved in hypogonadal men with type 2 diabetes. The subjects in this exploratory study were

relatively young in age, and with a brief duration of diabetes. It is possible that longer-term hypogonadotropism may lead to gonadal dystrophy not yet manifest in these men. It is also possible that the modest testicular stimulation induced by kisspeptin may not reveal deficiencies in maximal testosterone secretory potential elicited by hCG. Yet, it has to be noted that the levels of LH stimulation induced by kisspeptin-10 in type 2 diabetic males are in the physiological range. Moreover, our observation of preserved Leydig cell response in hypogonadal men with T2DM in the present study is in keeping with observations of normal hCG response in obese non-diabetic men (Vermeulen, Kaufman et al. 1993).

Decreased circulating serum LH concentrations observed in hypogonadal men with type 2 diabetes can arise from decreased LH pulse frequency (due to reduced GnRH pulse frequency), decreased pulse size (a function of GnRH secretory mass and pituitary gonadotrope sensitivity) or both. Baseline LH pulse frequency in our study subjects was lower than the pulse frequency reported in healthy men without diabetes while the secretory mass per LH pulse was comparable to that seen in healthy volunteers (chapter 3). This novel observation supports the notion that the inappropriately low concentrations of serum LH observed in men with type 2 diabetes (Dhindsa, Prabhakar et al. 2004; Dandona and Dhindsa 2011) may arise from a decrease in hypothalamic GnRH (and therefore LH) pulse frequency – as the link between decreased LH frequency and lower mean LH is well established (Spratt, Finkelstein et al. 1987). LH pulse frequency in obese men has previously been reported to be comparable to healthy volunteers, suggesting that the pathogenesis of hypogonadism in type 2 diabetes might be different from hypogonadism associated with obesity alone (Vermeulen, Kaufman et al. 1993). Because LH secretory burst-mass increased three-fold during the kisspeptin-10 infusion in men with diabetes, reduced GnRH secretion may also characterise diabetes. Whilst our results do not provide direct mechanistic explanations of this pathophysiological process, the observed normalisation of LH secretory parameters after exogenous administration of kisspeptin-10, taken together with the demonstration that kisspeptin antagonists in animal models decrease LH pulse frequency (Li, Kinsey-Jones et al. 2009; Roseweir, Kauffman et al. 2009; Millar, Roseweir et al. 2010) lends indirect support to the

hypothesis that hypogonadism in diabetes is mediated, at least in part, by reduced hypothalamic kisspeptin secretion (George, Millar et al. 2010).

Testosterone rapidly normalised during kisspeptin-10 infusion in our study with comparable Leydig cell responses to those seen in healthy male volunteers. However, longer term administration of kisspeptin-10 or its analogues is required to assess whether this increase can be sustained within the physiological range as tachyphylaxis of kisspeptin response has been reported in rodents (Roa, Vigo et al. 2008) and primates (Seminara, DiPietro et al. 2006; Ramaswamy, Seminara et al. 2007). A gradual decline in LH responsiveness to kisspeptin-54 has been reported in women when it was administered twice daily (Jayasena, Nijher et al. 2010). Twice-weekly kisspeptin-54 administration resulted in only partial desensitization, in contrast to the complete desensitisation observed with twice-daily administration (Jayasena, Nijher et al. 2010). However, desensitisation was not observed in the present study during 11 hours of infusion or in our previous studies involving intravenous infusion of kisspeptin-10 at 4 μg/kg/hr for 22.5 hours in healthy men (George, Veldhuis et al. 2011). Nevertheless, considering the phenotypic heterogeneity in men presenting with type 2 diabetes and hypogonadism (Dandona and Dhindsa 2011), longer studies with a larger number of participants are required to explore the pharmacological potential of kisspeptin analogues in hypogonadal men with diabetes.

As a potential therapeutic approach, there are theoretical advantages to the use of kisspeptin-mediated stimulation of endogenous testosterone over conventional testosterone replacement. Fertility is likely to be preserved as FSH secretion is maintained, unlike therapy with exogenous testosterone (Anderson and Baird 2002). Moreover, testosterone concentrations would be restored to the physiological range rather than raised to supraphysiological concentrations, as often observed with exogenous testosterone therapy which results in lowering of gonadotropin levels proportionately with serum testosterone (Swerdloff, Wang et al. 2000). The latter is particularly pertinent since there are safety concerns regarding the use of exogenous testosterone in individuals at high cardiovascular risk although there are small studies

reporting metabolic advantages to exogenous testosterone therapy in men with diabetes (Basaria, Coviello et al. 2010; Grossmann 2011).

Observations in the present study also have pharmacological implications for other types of male hypogonadism. Currently, central stimulation of the hypothalamic-pituitary axis is achieved with GnRH, delivered in a pulsatile manner to avoid pituitary desensitisation (Pitteloud, Hayes et al. 2002). This study provides the first demonstration that continuous rather than pulsatile kisspeptin-10 administration can achieve sustained LH stimulation in male hypogonadism, at least over the time period we have studied.

In conclusion, kisspeptin-10 evokes rapid and robust normalisation of LH pulse frequency and LH secretion in hypogonadal men with type 2 diabetes with resultant normalisation of circulating testosterone. These results suggest that the hypogonadism observed in men with type 2 diabetes is predominantly hypothalamo-hypophyseal in origin. These observations also suggest a potential therapeutic role for kisspeptin analogues in normalising endogenous testosterone secretion in hypogonadal men with type 2 diabetes.

8 Conclusions

In addition to the primary results discussed in chapters 3 to 8, there are also some additional observations – some of which potentially hypothesis generating- that can be made when the findings from two or more of the studies reported in this thesis are considered together.

8.1 Comparative stimulatory effect of GnRH vs. GnRH and Kisspeptin-10

In acute studies discussed in chapter 5, seven healthy male volunteers attended for two visits, at one of which, they were administered 100 μg of GnRH. From a baseline of 5.6±0.6 IU/L, LH increased by 36.4±3.9 IU/L to a peak of 42±4.1 IU/L. In comparison, when four healthy men with similar baseline LH (5.0±0.3 IU/L) were administered GnRH following a 22 hr infusion of kisspeptin-10, the peak LH observed was 66.2±2.1 IU/L. A two-sample *t* test demonstrates this increased responsiveness to GnRH co-administration to be statistically significant (P=0.001). In a volunteer who took part in both these studies, the peak LH observed following GnRH alone was 30.52 IU/L in comparison to 72.2 IU/L observed with co-administration of kisspeptin-10 and GnRH.

There are a few potential explanations for these observations, based on current knowledge – some more plausible than others. Firstly, the total amount of GnRH stimulating pituitary gonadotropes would be higher in the context of co-administration of GnRH - i.e., a sum of enhanced endogenous GnRH secreted and the 100 μg of exogenous GnRH would be available to stimulate LH secretion. Whilst this is the parsimonious explanation, it has to be noted that in dose-response studies of GnRH in men, near maximal stimulation of GnRH was observed with a 50 μg/kg dose, with doses as high as 250 μg and 500 μg not eliciting further stimulation (Snyder, Reitano et al. 1975). A 100 μg bolus dose is widely used as stimulatory test to assess maximal LH secretion in response to GnRH (Zevenhuijzen, Kelnar et al. 2004). It is therefore unlikely that endogenous GnRH secretion, although amplified with kisspeptinergic stimulation is likely to elicit an additive gonadotrophic response, unless there is an associated change in pituitary sensitivity. That GnRH has a self-

priming effect on pituitary gonadotropes is well established (Aiyer, Chiappa et al. 1974). An increase in pituitary sensitivity to pulsatile GnRH has also been demonstrated in patients with hypogonadotropic hypogonadism manifesting as delayed puberty (Smals, Hermus et al. 1994). However, increased GnRH responsiveness following pulsatile GnRH administration has not yet been demonstrated in eugonadal subjects. Nevertheless, these observations are consistent with the notion that there are two pools of LH in the human pituitary – one responding acutely to GnRH and the other responding to more prolonged stimulation by GnRH (Bremner and Paulsen 1974). Observations from men administered GnRH infusions demonstrating biphasic response in LH secretion – i.e. an early peak followed by late peaks support this notion (Bremner and Paulsen 1974).

8.2 Repetitive Kisspeptin-10 stimulation

Observations made in men with Kallmann Syndrome that kisspeptin-10 does not elicit LH stimulation raises the possibility that kisspeptin-10 could be used as a provocative diagnostic test to identify individuals with functional impairment of GnRH neuronal activity. However, for such a test to be of clinical use, the responses elicited in 'healthy' controls should be reproducible. As two volunteers had taken part in more than one clinical study involving kisspeptin-10 administration, we were able to compare the LH responses within the same individual when kisspeptin-10 was administered on different occasions. In three subjects administered 0.3 μg/kg kisspeptin-10 boluses twice, the peak LH observed was 2.7 times the baseline value (2.7±0.15 in studies described in the dose escalation studies described in chapter 3 and 2.7±0.4 in translational studies described in chapter 7) with a paired *t* test demonstrating statistical similarity (P = 0.99).

Another advantage of performing a kisspeptin stimulation test would be the remarkable similarity of proportionate stimulation from baseline values observed in individuals with diverse sex-steroid milieu, as described in chapter 4. Whilst these findings need to be reproduced in a larger cohort of individuals, these observations, when taken together with the fact that the current 'gold standard' provocative test, 100 μg GnRH test, elicits a wide range of responses in healthy volunteers - three to

ten-fold increase from baseline- (Apter, Cacciatore et al. 1989; Brito, Batista et al. 1999) suggests the diagnostic potential for a kisspeptin-10 provocative test.

8.3 Summary of findings

In conclusion, observations from the series of exploratory clinical studies reported in this thesis, would suggest that

- Kissppeptin-10 stimulates LH and FSH secretion in both men and women.
- Kisspeptin-10 stimulates LH secretion more than FSH secretion.
- Kisspeptinergic stimulation of LH secretion is only a fraction of maximal GnRH stimulated LH secretion.
- Kisspeptin-10 can stimulate LH secretion in women (including postmenopausal women).
- Kisspeptin-10 increases LH pulse frequency and LH pulse mass size – and thus GnRH pulse frequency by inference.
- GnRH signaling, but not Neurokinin B signaling, is obligatory for kisspeptinergic stimulation of gonadotropic secretion.
- Kisspeptin-10 can enhance testosterone secretion in men with hypogonadotropic hypogonadism associated with type 2 diabetes.

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Hypothesis: Kisspeptin Mediates Male Hypogonadism in Obesity and Type 2 Diabetes

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Key Words
Kisspeptin · Obesity · Metabolism · *KISS1* · *KISS1R* · GPR-54 · Diabetes · Metabolic syndrome · Hypogonadism · Testosterone

Abstract
Hypogonadism occurs commonly in men with type 2 diabetes (T2DM) and severe obesity. Current evidence points to a decreased secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus and thereby decreased secretion of gonadotropins from the pituitary gland as a central feature of the pathophysiology in these men. Hyperglycaemia, inflammation, leptin and oestrogen-related feedback have been proposed to make aetiological contributions to the hypogonadotropic hypogonadism of T2DM. However, the neuroendocrine signals that link these factors with modulation of GnRH neurons have yet to be identified. Kisspeptins play a central role in the modulation of GnRH secretion and, thus, downstream regulation of gonadotropins and testosterone secretion in men. Inactivating mutations of the kisspeptin receptor have been shown to cause hypogonadotropic hypogonadism in man, whilst an activating mutation is associated with precocious puberty. Data from studies in experimental animals link kisspeptin expression with individual factors known to regulate GnRH secretion, including hyperglycaemia, inflammation, leptin and oestrogen. We therefore hypothesise that decreased endogenous kisspeptin secretion is the common central pathway that links metabolic and endocrine factors in the pathology of testosterone deficiency seen in men with obesity and T2DM. We propose that the kisspeptin system plays a central role in integrating a range of metabolic inputs, thus constituting the link between energy status with the hypothalamic-pituitary-gonadal axis, and put forward potential clinical studies to test the hypothesis.

Introduction

Over the past few decades, obesity has become a global health challenge. Between 1980 and 2004 the prevalence of obesity increased from 15 to 33% in the United States – a pattern mirrored across the world [1]. Interlinked with this, the number of people with type 2 diabetes (T2DM) has also risen, and is predicted to rise further [2]. There is also an emerging consensus that T2DM and obesity are part of the same disease spectrum such that the term 'metabolic syndrome' is used to describe these overlapping pathophysiological processes [3].

Whilst the cardiovascular effects of T2DM and obesity have been extensively studied, the impact on fertility and hypogonadism remains relatively under-explored.

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Low testosterone concentrations in men have been shown to be associated with obesity and T2DM [4]. Plasma-free testosterone, total testosterone and sex hormone-binding globulin are low in obesity and recover in proportion to the degree of weight loss [5]. In men with established T2DM, decreased plasma-free testosterone is found in a third to half of all patients [6].

The relationship between hypogonadism and metabolic status, consistently seen across race and ethnic groups [7], also seems to be bidirectional. In obese men, biochemical hypogonadism (low testosterone) increases the risk of future development of diabetes [8]. In hypogonadal individuals with T2DM, treatment with testosterone improves the glucose and lipid profile [9], and enhances quality of life and cardiac performance [10].

The pathophysiology of low plasma testosterone in men with T2DM as well as the broader neuroendocrine signalling processes by which the metabolic milieu influences reproduction remains poorly understood [11]. However, the recent discovery of kisspeptin and its cognate receptor GPR-54 *(KISS1R)* as modulators of GnRH and, thus, downstream secretion of gonadotropins and testosterone [12] may help solve at least a part of this complex jigsaw.

Here we review human and animal experimental data on the potential role for kisspeptin in mediating metabolic inputs that modulate the hypothalamic-pituitary-gonadal (HPG) axis and put forward propositions for clinical studies to test this central role for kisspeptin as an integrator of metabolic cues with reproductive function in men with metabolic syndrome.

Hypothesis

We hypothesise that decreased endogenous kisspeptin secretion plays a central role in mediating hypogonadotropic hypogonadism in men with obesity and T2DM.

Hypogonadism in Obesity and Diabetes

A number of possible pathologies have been proposed as causative factors for hypogonadism in individuals with obesity and/or T2DM. Metabolic factors (hyperglycaemia, inflammation, oxidative stress) and testicular factors (decreased Leydig cell function) have all been proposed as factors contributing to the hypogonadal state in these individuals [4]. However, current evidence points to a decreased secretion of gonadotropin-releasing hormone (GnRH) from the hypothalamus as the key factor [4], as evidenced by the low or relatively low luteinising hormone (LH) and follicle-stimulating hormone (FSH) concentrations in the large majority of these men with low plasma testosterone. LH pulse amplitude and secretory masses are significantly lower in obese men than in lean controls [13]. Moreover, there is a highly significant negative correlation between plasma-free testosterone and body mass [13]. A normal LH and FSH response to GnRH has also been demonstrated in obese individuals, suggesting a hypothalamic rather than a pituitary defect. [14].

The Role of Kisspeptin in GnRH Secretion

Kisspeptins are peptide products of the *KISS1* gene [15]. Various forms of kisspeptin have been identified, all of which have the same decapeptide amino acid sequence at the C-terminus [12]. Kisspeptins regulate the reproductive axis by stimulation of their cognate receptor *KISS1R* (also called GPR-54) on GnRH neurons, thereby regulating LH and FSH secretion [12]. Administration of kisspeptin has been shown to increase plasma LH, FSH and testosterone in a dose-dependent manner in a number of species including man [16], and a central role of kisspeptin in the metabolic regulation of reproductive function is well-recognised [17]. Patients with mutations of *KISS1R* exhibit hypogonadotropic hypogonadism but normal responses to GnRH and no reported metabolic derangement [18–20]. It is also important to note that metabolic abnormalities have not been reported in knockout models lacking kisspeptin or its receptor [21], suggesting that perturbation in the kisspeptin system is more likely to be the effect rather than the cause of a metabolic phenotype.

In rodents, two distinct populations of kisspeptin neurons exist in the arcuate (ARC) and anterior periventricular (AVPV) nuclei, with differential functional responses [22]. Kisspeptin neurons in the ARC have been shown to respond to negative feedback by gonadal steroids, while AVPV kisspeptin neurons in the female rodent respond to positive feedback by oestrogen [22]. However, AVPV is not an anatomical area present in the human hypothalamus and no kisspeptin neurons have been demonstrated in the corresponding rostral periventricular region of the third ventricle in humans [22]. The infundibular nucleus is the human homologue of the ARC where the presence of kisspeptin neurons has been documented [23]. In the rodent data reviewed here, we will focus on the kisspeptin population in the ARC nucleus as the oestrogen-mediated positive feedback seen in the female is not of particular relevance in the context of male hypogonadism. However, it has to be borne in mind that in mRNA analyses of

gene expression, whole hypothalami are often used rather than specific nuclei, such as the ARC or AVPV, thus potentially lowering the sensitivity and specificity of any changes detected in Kiss1 mRNA expression.

Support for the Hypothesis

Oestrogen-Mediated Negative Feedback and Kisspeptin

Peripheral conversion of androgens to oestrogens in obese men leads to increased circulating oestrogens, which in turn suppress the hypothalamic-pituitary axis and thus the production of testosterone [24]. Serum oestrone and 17-β-estradiol are elevated twofold in morbidly obese men, with the urinary excretion rates of these hormones elevated in proportion to the degree of obesity [24]. Treatment with an aromatase inhibitor (preventing conversion of testosterone to estradiol) produces a sustained normalisation of serum total testosterone in obese men with hypogonadism [25].

However, adult GnRH neurons lack oestrogen receptor-α (ER-α) and rely on signaling from other oestrogen responsive hypothalamic neurons to facilitate oestrogen-mediated regulation [26]. The phenotypic identity of cells that receive input from gonadal steroids and relay this information to GnRH neurons remains unknown; however, kisspeptin neurons appear likely to play this role [27]. The hypothalamic content of *Kiss1* (mRNA) increases significantly after gonadectomy and decreases with sex steroid replacement [28]. Kisspeptin neurons in sheep express ER-α receptors [29] and in mouse models that lack functional ER-α receptors, *Kiss1* mRNA expression does not change in response to estradiol treatment [30]. A minority of kisspeptin neurons express the oestrogen receptor isoform ER-β in addition to ER-α [30]. In the absence of ER-β (in knockout mouse models), the inhibitory effect of E2 on transcription of *Kiss1* is enhanced [30], indicating functional roles of both ER types in the regulation of kisspeptin neurons.

Leptin and Kisspeptin

Leptin secreted from adipocytes conveys information about the body's energy stores to the brain and thus regulates a number of physiological processes, including inflammation, immune function and reproduction [31]. Humans and mice lacking leptin (ob/ob mice) or leptin receptor (db/db mice) develop obesity, insulin-resistant diabetes and hypogonadism [31]. In obese men, circulating androgen is inversely correlated with circulating leptin [32] and a functional resistance to leptin in human obesity has been described [33].

GnRH neurons, however, show little or no expression of Ob-R mRNA [34] and, therefore, must rely on intermediary neurons for transmission of information on leptin concentrations [34]. Kisspeptin neurons may fulfill this role, as they express leptin receptors [35]. Reversal of fasting-induced inhibition of gonadotropins by exogenous kisspeptin [36] and the ability of intracerebroventricular kisspeptin administration to correct the decrease in LH secretion seen in pubertal animals after central immunoneutralisation of endogenous leptin with antileptin antibody [37] provide further evidence for a role of kisspeptin in affecting the influence of leptin on the HPG axis.

Hypothalamic *Kiss1* mRNA expression is significantly reduced in ob/ob compared to wild-type mice and are increased in ob/ob mice treated with leptin [35, 38]. Moreover, administration of leptin increases hypothalamic *Kiss1* mRNA as well as LH and testosterone concentrations in hypogonadotropic diabetic male rats [38].

Hyperglycaemia and Kisspeptin

Plasma testosterone is inversely related to glycaemic control in men with T2DM. However, when compared with age- and BMI-matched men [4] as well as men with type 1 diabetes of a comparable age, men with T2DM have a higher prevalence of hypogonadism [39]. This suggests insulin resistance as well as hyperglycaemia could also play a role in the pathogenesis of hypogonadism in this setting [4]. Mice with neuron-specific deletion of insulin receptors (NIRKO mice) have increased body mass and hypogonadotropic hypogonadism, confirming that insulin sensing in the brain is required for normal activity of the HPG axis [40].

Hypothalamic *Kiss1* mRNA is decreased in streptozotocin-induced diabetic rats (a model of type 1 diabetes), and the post-orchidectomy rise in *Kiss1* mRNA in these hyperglycaemic animals is severely blunted [38]. Kisspeptin administration has been shown to evoke robust LH and testosterone secretion and enhance post-gonadectomy LH concentrations in hypogonadotropic streptozotocin-induced diabetic male rats [38]. Repeated administration of kisspeptin to diabetic rats also partially rescued prostate and testis weights [41]. However, no studies have yet been carried out on *Kiss1* mRNA expression in animal models of T2DM.

Inflammation and Kisspeptin

Obesity and T2DM are now well recognised to be associated with inflammation and increased oxidative

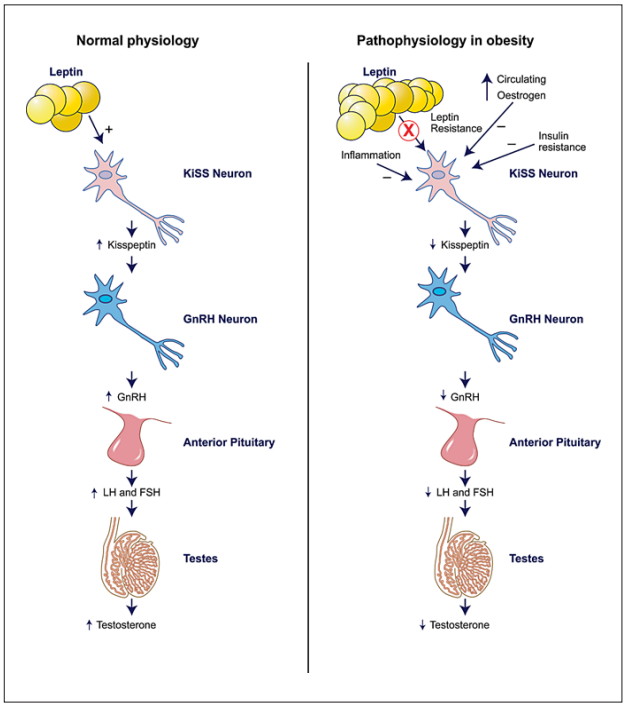

Fig. 1. Normal physiology and the pathophysiological processes underpinning our hypothesis.

stress [42]. Circulating C-reactive protein, an inflammatory marker associated with cardiovascular risk, has been shown to be significantly higher in hypogonadal men with T2DM in comparison to men with normal plasma testosterone [4].

There are emerging data linking kisspeptin and inflammation. Administration of a pro-inflammatory agent (lipopolysaccharide) decreased hypothalamic *Kiss1* mRNA expression as well as plasma LH in ovariectomised rats [43]. Indomethacin, an anti-inflammatory drug, completely blocked both these suppressive effects [43]. Whilst inflammation has long been recognised to suppress the reproductive axis (e.g. in severe or chronic illness), these early findings suggest a link between kisspeptin and inflammation.

Figure 1 summarises the normal physiology and the proposed pathophysiology underpinning our hypothesis.

Testing the Hypothesis

To test the hypothesis directly, comparison between endogenous kisspeptin secretion in obese and lean subjects would have to be made. However, the concentration of kisspeptin entering the peripheral circulation from the hypothalamus is likely to be undetectable. Accessing portal circulation via petrosal sinus sampling for sampling is technically and ethically challenging. Highly specific and sensitive assay methods to measure kisspeptins are not yet widely available [44, 45]. Moreover, even if the technical barriers are overcome, circulatory concentrations of kisspeptin may not correlate with the intracranial milieu.

Recent advances in kisspeptin biology offer indirect alternatives to test our hypothesis. Kisspeptin neuronal hypertrophy and increased kisspeptin gene expression has been demonstrated in the hypothalamic infundibular nu-

cleus in postmenopausal women [23]. Similar post-mortem histological analysis of hypothalami from obese men may show hypotrophy of kisspeptin neurons and/or decreased *KISS1* expression. A comparable situation of hypogonadotropic hypogonadism is seen in women with hypothalamic amenorrhoea who show a significantly higher LH response to acute administration of kisspeptin-54 in comparison to healthy women in the follicular phase of menstrual cycle [46]. As hypogonadal men with metabolic syndrome have a similar gonadotropin profile to women with hypothalamic amenorrhoea, stimulatory responses in LH to exogenous kisspeptin in these men may also be increased, which if confirmed in exploratory studies, raises the possibility of using a provocative kisspeptin stimulation test as a marker of metabolic status. In recent animal studies, kisspeptin antagonists have been shown to inhibit kisspeptin-induced LH release and also block the post-gonadectomy rise in LH [47]. Moreover, in male rats with streptozocin-induced diabetes, the LH response to kisspeptin was significantly higher than control animals [48]. By analogy, increased sensitivity to kisspeptin analogues in men with hypogonadism associated with T2DM would indirectly lend support to our hypothesis. Such studies could be performed by acute stimulation with a single dose of kisspeptin, akin to the GnRH provocative test used in numerous clinical settings.

Implications of the Hypothesis

There are physiological and clinical implications if our postulates are correct.

The pathophysiology of hypogonadism in obese men remains poorly understood. Current evidence linking insulin, leptin, oestrogen and inflammation with hypogonadism fails to explain how these factors have effects on GnRH neurons which lack corresponding receptors. If our hypothesis is correct, kisspeptin will constitute the neuroendocrine link conveying information about whole body energy balance to the GnRH neuronal system.

From a translational perspective, there is potential for pharmacological therapies to be developed to address hypogonadism. A number of kisspeptin analogues are being developed with agonistic and antagonistic activities. If our hypothesis is correct, kisspeptin agonists constitute putative therapeutic targets for low circulating testosterone seen in obesity and diabetes. Current treatment of hypogonadism with testosterone tends to decrease sperm production through negative feedback on FSH secretion with resultant sub-fertility in at least some patients. Pharmacological modulation with kisspeptin agonists will potentially not have this adverse effect.

In summary, we postulate low kisspeptin secretion is part of the pathophysiology of hypogonadotropic hypogonadism seen in men with obesity and T2DM. The kisspeptin/KISS1R system is likely to be a central link between energy status and the HPG axis in man as has been demonstrated in experimental animals.

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Kisspeptin-10 Is a Potent Stimulator of LH and Increases Pulse Frequency in Men

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Context: Kisspeptins stimulate GnRH and thus gonadotropin secretion. Kisspeptin-10 is the minimal kisspeptin sequence with full intrinsic bioactivity, but it has not been studied in man.

Objective: We investigated our hypothesis that kisspeptin-10 increases GnRH and thus LH pulse frequency.

Design and Participants: The dose response of kisspeptin-10 was investigated by administering iv bolus doses (0.01–3.0 μg/kg) and vehicle to healthy men. Effects on LH pulse frequency and size were determined by deconvolution analysis during infusion of kisspeptin-10 for up to 22.5 h.

Results: Intravenous bolus kisspeptin-10 resulted in a rapid and dose-dependent rise in serum LH concentration, with maximal stimulation at 1 μg/kg (4.1 $\pm$ 0.4 to 12.4 $\pm$ 1.7 IU/liter at 30 min, $P <$ 0.001, n = 6). Administration of 3 μg/kg elicited a reduced response *vs.* 1 μg/kg ($P < 0.05$). Infusion of kisspeptin-10 at 4 μg/kg $\cdot$ h for 22.5 h elicited an increase in LH from a mean of 5.4 $\pm$ 0.7 to 20.8 $\pm$ 4.9 IU/liter (n = 4; $P < 0.05$) and serum testosterone increased from 16.6 $\pm$ 2.4 to 24.0 $\pm$ 2.5 nmol/liter ($P < 0.001$). LH pulses were obscured at this high rate of secretion, but a lower dose infusion of kisspeptin-10 (1.5 μg/kg $\cdot$ h) increased mean LH from 5.2 $\pm$ 0.8 to 14.1 $\pm$ 1.7 IU/liter (n = 4; $P < 0.01$) and increased LH pulse frequency from 0.7 $\pm$ 0.1 to 1.0 $\pm$ 0.2 pulses/h ($P < 0.05$) and secretory burst mass from 3.9 $\pm$ 0.4 to 12.8 $\pm$ 2.6 IU/liter ($P < 0.05$).

Conclusions: Kisspeptin-10 boluses potently evoke LH secretion in men, and continuous infusion increases testosterone, LH pulse frequency, and pulse size. Kisspeptin analogues have therapeutic potential as regulators of LH and thus testosterone secretion. (***J Clin Endocrinol Metab* 96: E1228–E1236, 2011**)

The central role of GnRH (LHRH) in regulating reproduction by stimulating the secretion of pituitary gonadotropins (LH and FSH) is well established (1). Kisspeptin, a hypothalamic neuropeptide encoded by the *KISS1* gene, has recently emerged as a key central regulator of GnRH secretion. Kisspeptin signaling is obligatory for normal pubertal maturation, as evidenced by absent or advanced pubertal development in individuals with mutations in genes encoding kisspeptin (2) and its receptor [KISS1R; also known as G protein-coupled receptor (GPR) 54] (3–6). Administration of exogenous kisspeptin stimulates LH secretion in both men and women (7–9).

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Abbreviations: AUC, Area under the curve; GnIH, gonadotropin inhibitory hormone; GPR, G protein-coupled receptor; KISS1R, kisspeptin receptor.

Human studies of kisspeptin have hitherto used the 54-amino acid peptide, kisspeptin-54 (7–10). Kisspeptin-54 is cleaved from the 145-amino acid precursor polypeptide encoded by *KISS1* and is further processed to 14, 13, and 10 amino acid (kisspeptin-10) sequences, all sharing the same C-terminal decapeptide RFAmide (arginine-amidated phenylalanine) sequence (11). Although kisspeptin-10 has intrinsic bioactivity similar to the longer kisspeptin fragments (11), it is also characterized by a shorter half-life and more rapid onset of action after iv administration in rodents (12). Kisspeptin-10 also has greater potential for pharmaceutical development because both agonists (13) and antagonists (14, 15) have been developed based on its decapeptide sequence. However, there have been no studies on the activity of kisspeptin-10 in humans.

Alterations in GnRH pulsatility and thus LH secretion are a feature of a number of reproductive disorders. Individuals with some forms of male hypogonadism (16) and hypothalamic amenorrhea (17) show decreased pulse frequency, whereas women with polycystic ovarian syndrome show an increase (18). However, neuroendocrine mechanisms underpinning GnRH pulse generation are yet to be fully delineated (19). Experimental animals exposed to kisspeptin antagonists demonstrate decreased LH pulsatile secretion, suggesting that kisspeptin modulates GnRH pulse frequency (20). Although administration of kisspeptin-54 acutely stimulates LH secretion in men and women (7, 8), it is unclear whether this is mediated by a change in LH pulse frequency, which would indicate an underlying stimulatory effect on GnRH pulse frequency.

In these first-in-human studies of kisspeptin-10, we aimed to establish the dose dependency and time course of stimulation of LH secretion after iv bolus doses of kisspeptin-10 and to compare the magnitude of this stimulated LH secretion with that after a maximally stimulatory dose of GnRH. Having established that kisspeptin-10 is an effective LH secretagogue, we further investigated the effects of infusion of kisspeptin-10 on LH pulsatility. We also examined whether high-dose continuous infusion of kisspeptin-10 induces tachyphylaxis of LH response, as previously suggested in a male primate model (21).

Participants and Methods

Participants

Six healthy male volunteers took part in acute kisspeptin stimulation studies and four healthy males in each of the infusion studies. All volunteers provided informed written consent, and the study was approved by a local research ethics committee (Lothian Research Ethics Committee References 09/S1101/23 and 09/S1101/67). The age of healthy male participants was 35.6 ± 3.4 (mean ± SEM) and body mass index was 26.1 ± 0.8 kg/m^2, all of whom had a minimum bilateral testicular volume of 40 ml, normal physical examination, and normal secondary sexual characteristics. Baseline full blood count, renal function, liver function, and electrolytes were within normal limits.

Study drugs

Kisspeptin-10 was custom synthesized under GMP standards (Bachem GmBH, Weil am Rhein, Germany). Purity was assessed by HPLC at 97% with a mass balance of 98.8%. For each participant, kisspeptin-10 was made up within the hour before injection by diluting 1 mg of lyophilized kisspeptin-10 in 5 ml sterile normal saline. Stability of kisspeptin-10 in solution was assessed by *in vitro* receptor binding studies comparing preincubated and freshly constituted 0.2 mg/ml solution (data not shown). A bolus of 0.5 ml sterile normal (0.9%) saline was injected as vehicle. Commercially available GnRH (Relefact; Sanofi Aventis, Frankfurt, Germany) was used for GnRH stimulation.

Protocols

Kisspeptin-10 dose response study

This study was designed to assess the magnitude and dose dependency of gonadotropin secretion in response to six iv bolus doses (0.01, 0.03, 0.1, 0.3, 1.0, and 3.0 μg/kg) of kisspeptin-10 or vehicle (normal saline).

All volunteers attended the study in a fasting state, and all visits commenced between 0800 and 0900 h to avoid diurnal bias. Blood samples were obtained through an indwelling iv cannulae before (60, 45, 30, 20, and 10 min and immediately) and after (10, 20, 30, 45, 60, 75, and 90 min) kisspeptin-10 or vehicle administration. Doses were administered in increasing order for safety reasons and visits were at least 1 wk apart. All volunteers received at least four doses and four volunteers received all doses. Participants were blinded to the dose of kisspeptin-10.

Blood pressure, pulse, and peripheral oxygen saturations were measured every 3 min with standard automated techniques. Full blood count, serum electrolytes, liver function, and renal function were checked at each visit.

High-dose kisspeptin-10 iv infusion

This study was designed to assess LH secretion and potential desensitization of kisspeptin-KISS1R with a continuous high-dose infusion of kisspeptin-10.

After an overnight fast, four volunteers attended our clinical research facility for a 34-h supervised stay. Blood samples were collected at 10-min intervals for two 12-h periods on consecutive days and hourly overnight. After 9 h of baseline sampling, 3 μg/kg of kisspeptin-10 (the highest dose previously investigated) was administered as an iv bolus and a continuous iv infusion of kisspeptin-10 (Crono PCA; Cane, Rivoli, Italy) commenced 90 min later at 4 μg/kg · h. This infusion rate, maintained for 22.5 h, was selected to aim to give a comparable hourly dose to the maximally effective bolus doses, bearing in mind the expected short half-life of kisspeptin-10. A 100-μg/kg iv bolus of GnRH was administered 60 min before the end of the infusion.

Serum LH during the 9-h baseline frequent sampling period was compared with the corresponding 9-h frequent sampling period on the second day of the study during the iv infusion of kisspeptin-10. In addition, we compared the mean LH values observed in the initial 90 min of kisspeptin-10 infusion with the

mean LH values observed in the final 90 min of infusion before GnRH administration to identify any potential tachyphylaxis.

Comparisons were also made between mean LH over the 60 min before GnRH administration and the peak LH 30 min afterward. Serum testosterone was analyzed at 30-min intervals during the two 9-h frequent sampling periods before and during the infusion.

All subjects were provided with standardized meals throughout the study and a period of overnight fast maintained between 2200 and 0900 h. Full blood count, electrolytes, liver function, and renal function were checked at the beginning of the study, 24 h later, and at the end of the study.

Pulse frequency study

As there was an apparent increase in LH pulse frequency during the high-dose kisspeptin-10 infusion, we examined this further using a lower dose of kisspeptin-10.

Four healthy men attended our clinical research facility for two visits 5 d apart for 10-min blood sampling. At the first visit, baseline LH pulsatility was assessed over a 9-h period. During the second visit, an iv infusion of kisspeptin-10 was administered for 9 h at 1.5 μg/kg · h after an hour of baseline sampling. Subjects were provided with standardized meals during the study visits.

Hormone assays

Blood samples were centrifuged immediately at 4 C for 10 min at 3000 rpm and serum frozen at −20 C until analysis. LH and FSH were determined by ELISA and testosterone by RIA as previously described (22). Interassay coefficient of variation for all hormonal assays was less than 5% at the concentrations measured. Intraassay coefficient of variation of LH was 2.9%. All samples from each of the study visits were analyzed together in duplicate.

Statistical analysis

For the dose-finding study, 60-min mean LH and FSH concentrations and area under the curve (AUC; by trapezoid integration) before and after the kisspeptin-10 bolus were calculated. Variance in mean LH and ΔAUC values in the dose-response study was analyzed by ANOVA followed by Tukey's simultaneous test. Student's *t* test was used to analyze changes in mean LH, FSH, and testosterone concentrations in the infusion studies.

LH pulses were identified using a deconvolution algorithm using cluster analysis with 93% sensitivity and specificity on blinded data and basal, pulsatile, and total LH secretion calculated (23). Paired Student's *t* test was used to assess changes in pulse frequency.

Data are presented as mean ± SEM. A two-sided $P < 0.05$ was regarded as statistically significant for all analyses. The statistical software package Minitab 16 (Minitab Ltd., Coventry, UK) was used.

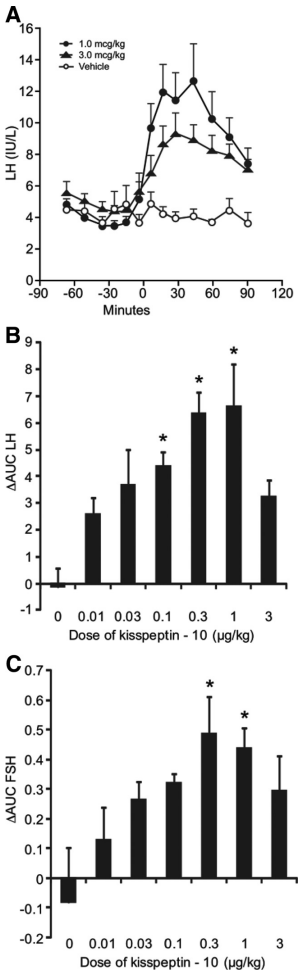

FIG. 1. Gonadotropin response to acute administration of kisspeptin-10 to normal men (n = 6). A, Serum LH concentration in response to 1 μg/kg kisspeptin-10 (*filled circles*), 3 μg/kg kisspeptin-10 (*filled triangles*), or vehicle (*open circles*) administered to healthy volunteers at time 0. B, Δ AUC (60 min) of LH after kisspeptin-10 and vehicle administration. C, Δ AUC (60 min) of FSH after kisspeptin-10 and vehicle administration. Mean ± SEM. *, $P < 0.05$ *vs.* vehicle.

Results

Kisspeptin-10 dose-response study

Intravenous injections of kisspeptin-10 elicited a rapid increase in LH in all volunteers, with peak concentrations seen by 45 min after injection for all doses studied. The maximum LH stimulation was seen after the 1-μg/kg bolus, achieving peak (12.4 ± 1.7 IU/liter) concentration at 30 min (Fig. 1A).

There was a clear dose-dependent increase in LH concentrations in response to kisspeptin-10 ($P < 0.0001$). Statistically significant increases were observed after the 0.03-, 0.1-, 0.3-, and 1-μg/kg bolus doses ($P <$

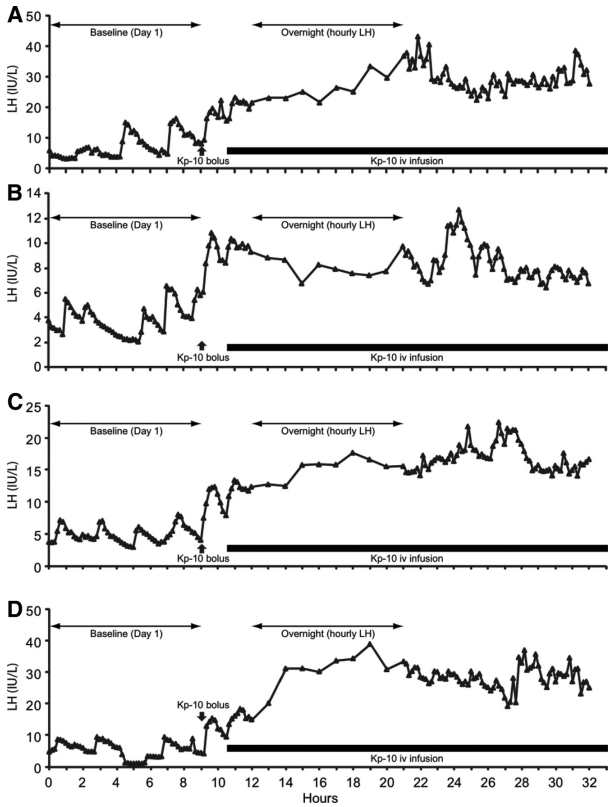

FIG. 2. Serum LH profiles of four individual subjects receiving 4 μg/kg · h kisspeptin-10 infusion (*black bar*) after 9 h baseline sampling and 3 μg/kg iv bolus administered at 9 h (*black arrow*). Serum samples were obtained at 10 min intervals except overnight when samples were obtained hourly. Note the difference in scale between individual subjects.

0.01). There was, however, no significant increase in LH concentration after the highest dose administered (3.0 μg/kg), and the mean LH after this dose was significantly less than after the 0.3- and 1-μg/kg doses (both $P < 0.05$). Calculation of the AUC over 60 min following the kisspeptin-10 administration showed a similar pattern, with the 0.1-, 0.3-, and 1-μg/kg doses achieving statistically significant changes compared with vehicle (Fig. 1B).

FSH secretion also increased dose dependently after kisspeptin-10 administration ($P = 0.012$). The largest increase was observed with 1 μg/kg in which the mean 60-min FSH rose from 4.6 ± 1.0 to 5.3 ± 1.0 IU/liter. Increases were seen in the AUC of FSH after 0.3 and 1 μg/kg kisspeptin-10, with 3 μg/kg again not resulting in a significant increase in FSH concentration (Fig. 1C). Serum testosterone concentrations showed no statistically significant change with any of the doses studied (data not shown).

Blood pressure, heart rate, peripheral oxygenation, liver function, renal function, hemoglobin, mean corpuscular volume, and electrolytes remained stable in all subjects throughout the study period. No adverse events were reported.

Kisspeptin-10 continuous infusion studies

Infusion of 4 μg/kg · h kisspeptin-10 resulted in sustained increases in LH concentration. The LH profiles observed in the four individual study subjects are represented in Fig. 2. A bolus injection of 3 μg/kg kisspeptin-10 resulted in an increase in LH to 13.6 ± 1.7 IU/liter ($P < 0.05$ *vs.* baseline) at 30 min. Continuous kisspeptin-10 infusion (4 μg/kg · h) resulted in a further increase in LH secretion in all subjects, which was sustained throughout the 22.5 h of the infusion. Mean LH during kisspeptin-10 infusion was 20.9 ± 4.9 *vs.* 5.5 ± 0.8 IU/liter over the 9-h pretreatment period ($P < 0.05$, Fig. 3A).

Mean LH concentrations in the final 90 min of infusion (23.9 ± 6.8 IU/liter) was comparable with that in the first 90 min of infusion (16.3 ± 2.8 IU/liter, $P = 0.34$). These data therefore do not indicate tachyphylaxis of the LH response to kisspeptin-10 administration at this dose and duration of exposure.

Intravenous administration of 100 μg bolus of GnRH 60 min before the end of the kisspeptin-10 infusion increased LH further with a peak of 89.3 ± 6.1 IU/liter. This stimulatory effect of GnRH was at least 2.5-fold greater in all subjects than the peak LH observed with the iv bolus of kisspeptin-10 (13.6 ± 1.7 IU/liter, $P < 0.001$) and the peak LH achieved during kisspeptin-10 infusion (28.7 ± 7.0 IU/liter, $P < 0.01$).

Analysis of LH pulse frequency during the 9-h baseline sampling and the corresponding 9-h period on the second day of infusion was performed. Characteristic pulsatile LH secretion was observed in all four subjects at baseline but was not well defined during kisspeptin-10 infusion.

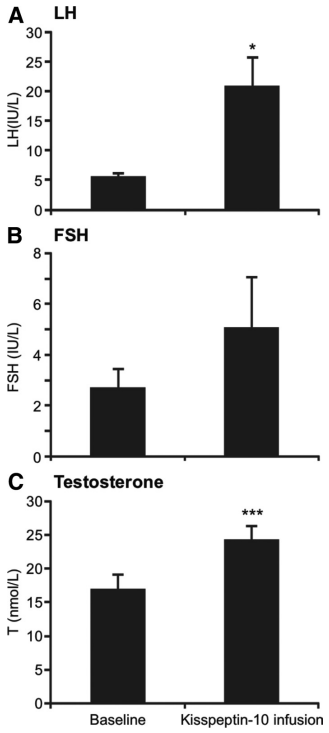

FIG. 3. Gonadotropin and testosterone response to infusions of 4 μg/kg of kisspeptin-10 (n = 4). A, Mean serum LH before and during kisspeptin-10 infusion. B, Mean serum FSH before and during kisspeptin-10 infusion. C, Mean serum testosterone before and during kisspeptin-10 infusion. *Error bars* represent SEM. *, $P < 0.05$; ***, $P < 0.001$.

Nevertheless, deconvolution analysis showed an in increase in pulse frequency in three subjects (from 0.4 to 0.6, 0.6 to 0.8, and 0.6 to 1.2 pulses/h, Fig. 2, A–C, respectively), whereas one subject showed a slight decrease from 0.7 to 0.6 pulse/h (Fig. 2D), giving a mean pulse frequency of 0.6 ± 0.1 pulses/h at baseline and 0.8 ± 0.2 during kisspeptin-10 infusion (P = ns).

Although FSH showed an increase in all subjects during infusion of 4 μg/kg · h kisspeptin-10, this was not statistically significant (2.7 ± 0.8 to 5 ± 2.0 IU/liter, $P = 0.17$, Fig. 3B), whereas testosterone concentration significantly increased from 16.6 ± 2.4 to 24.0 ± 2.5 nmol/liter ($P < 0.001$, Fig. 3C).

To investigate further whether continuous kisspeptin-10 exposure increases LH pulse frequency, we infused a lower dose of 1.5 μg/kg · h for 9 h. Mean LH increased significantly from 5.2 ± 0.8 IU/liter at baseline to 14.1 ± 1.7 IU/liter during kisspeptin-10 infusion ($P < 0.01$, n = 4, Fig. 4). LH showed a steady increase throughout the course of the infusion, increasing from 7.2 ± 2.3 IU/liter in the first 90 min of infusion to 20.4 ± 3.5 IU/liter in the last 90 min ($P < 0.05$, Fig. 4).

Deconvolution analysis demonstrated an increase in LH pulse frequency in all subjects, with a mean increase from 0.7 ± 0.1 to 1.0 ± 0.2 pulses/h ($P = 0.01$) during kisspeptin-10 infusion at 1.5 μg/kg · h (Fig. 4E). Secretory mass of LH per pulse also increased in all subjects during the infusion, from 3.9 ± 0.4 to 12.8 ± 2.6 IU/liter ($P < 0.05$, Fig 4F). Pulsatile LH secretion increased as a proportion of total LH secretion in all subjects from 41.3 ± 5.7 to 69.5 ± 5.9% during the infusion.

Serum FSH also increased progressively during the lower-dose kisspeptin-10 infusion. FSH observed during the final 90 min of the infusion (6.6 ± 0.8 IU/liter) showed a significant increase from the first 90 min (3.8 ± 0.8 IU/liter, $P < 0.05$) of the infusion and baseline (3.9 ± 0.7 IU/liter, $P < 0.05$).

Discussion

We have demonstrated that iv administration of kisspeptin-10 boluses result in potent and dose-dependent stimulation of LH secretion in healthy men. Doses of kisspeptin-10 as low as 0.03 μg/kg (23 pmol/kg) elicited a significant rise in LH when compared with vehicle, demonstrating the high potency of kisspeptin-10. This is in keeping with data from experimental animals where intracerebroventricular doses of kisspeptin as small as 1 fmol effected robust LH stimulation (24). Central rather than pituitary mediation of the effect of kisspeptin on LH secretion has been demonstrated by abolition of the effect of kisspeptin by pretreatment with a GnRH antagonist (24–26).

The 2.5-fold increase in serum LH from baseline observed after kisspeptin bolus administration in our study is comparable with that observed in men administered equimolar doses of kisspeptin-54 as short infusions (7). Moreover, as in human studies using kisspeptin-54 (7), the stimulatory effect of kisspeptin-10 on LH secretion in our study was also markedly more pronounced than that on FSH secretion. Early animal studies suggested stimulatory effects on LH and testosterone secretion were seen only with the longer kisspeptin peptide, kisspeptin-54, and not shorter forms (27). However, *in vivo* rodent data have subsequently shown equal potency of kisspeptin-10 and kisspeptin-54 (24, 28), and in *in vitro* binding assays, both peptides show equal affinity at the KISS1R (11). Our re-

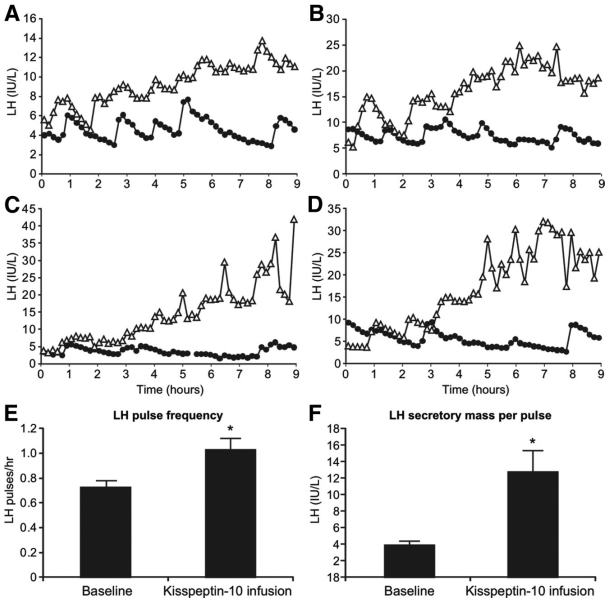

FIG. 4. Gonadotrophin response to 9-h iv infusion of 1.5 μg/kg kisspeptin-10 (n = 4). A–D, LH profiles from individual subjects during baseline (*closed circles*) and kisspeptin-10 infusion (*open triangles*) visits. Note the difference in scale between individual subjects. E, Mean LH pulse frequency during baseline and kisspeptin-10 infusion visits. F, Secretory mass of LH per pulse during baseline and kisspeptin infusion visits. *Error bars* represent SEM. *, $P < 0.05$.

sults demonstrate that the 10 amino acid form of kisspeptin is a potent LH secretagogue *in vivo* in humans.

Although there was a clear dose dependency of LH response to increasing doses of kisspeptin-10 up to 1 μg/kg, the 3-μg/kg (2.3 nmol/kg) bolus dose did not elicit a statistically significant increase when compared with vehicle, and mean and peak LH concentration after this dose was lower than corresponding values after 0.3 and 1 μg/kg. This phenomenon has not been reported before after bolus administration of kisspeptin, but continuous infusion of kisspeptin-10 in primates (21) and repeated sc administration of kisspeptin-54 in human subjects with hypothalamic amenorrhea (9) resulted in tachyphylaxis. The kisspeptin receptor KISS1R is known to desensitize rapidly *in vitro* (29), raising the possibility of rapid hypothalamic desensitization. Although such desensitization is a parsimonious explanation for the decreased response observed here, desensitization has to be occurring rapidly after kisspeptin-10 administration before maximal stimulation of gonadotrophs is achieved. Because the fast half-life of LH in healthy men is 18 min (30), lower peak LH after the 3 μg/kg would suggest that the gonadotroph stimulation was submaximal.

An alternative explanation for this observation is that at high concentrations, kisspeptin-10 may stimulate another RF-amide receptor with an inhibitory effect on GnRH or LH secretion. Kisspeptin-10, at nanomolar concentrations, has been shown to bind and activate the gonadotropin inhibitory hormone (GnIH) receptor [GPR147, NPFFR1 (neuropeptide FF receptor 1)] (31), which is expressed both in the hypothalamus and on gonadotropes (32). Nanomolar plasma concentrations of kisspeptin were achieved after sc administration of doses of kisspeptin-54 similar to the kisspeptin-10 used in our study (8). However, although peak plasma kisspeptin-10 concentrations after the 3-μg/kg bolus may have been sufficient to activate GnIH receptors, there is little evidence for a functional role for GnIH in humans, and *in vivo* studies in sheep (33) would suggest that its effects are relatively modest, and it is not possible to exclude an effect of GnIH receptor involvement in the infusion studies. The reduced stimulatory efficacy of kisspeptin-10 at high concentrations demands careful dosing in any potential therapeutic applications.

Importantly, our studies demonstrate the novel phenomenon that continuous infusion of kisspeptin-10 increases LH pulse frequency. Along with increased secretory mass in individual pulses, this increase in pulse frequency resulted in pulsatile secretion contributing to a higher proportion of total LH secreted. Data from other investigators have not shown that GnRH pulse frequency is increased by an acute bolus of kisspeptin (34), indicating the need for continuing kisspeptin stimulation to study its influence on GnRH pulse frequency. The dependency of LH pulsatility on pulsatile GnRH secretion (1, 35) is well established, and kisspeptin has been shown to directly stimulate GnRH secretion (36) with electrophysiological studies showing intense and prolonged activation of rodent GnRH neurons by kisspeptin-10 (37). There is a marked stochastic increase in LH when kisspeptin is applied to the hypothalamic arcuate nucleus in rodents, which might reflect an acceleration of LH pulse frequency (20), and a kisspeptin antagonist decreases LH pulse frequency in this model (20). Systemic administration of kisspeptin-10 in our study prevents us from concluding whether this increase in LH (and by inference GnRH)

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