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Kisspeptin and neurokinin B in the regulation of the human hypothalamic-pituitary-gonadal axis

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Declaration

I declare that

- This thesis has been composed by me;
- The work submitted is my own, except where work has formed part of jointly authored publication. My contributions and those of others to this thesis are indicated below;
- No part of this thesis has been submitted for any other degree or professional qualification.

Prof Richard A Anderson and Dr Jyothis T George were my supervisors.

Karolina Skorupskaite
March 2017

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Abstract

Background: Hypothalamic kisspeptin and neurokinin B (NKB) are central regulators of GnRH and thus gonadotropin (LH and FSH) secretion. Men and women with loss-of-function mutations in NKB-kisspeptin pathway show hypogonadotropic pubertal delay with reduced GnRH/LH pulsatility. Studies in patients with defects in NKB signalling suggest that kisspeptin is functionally downstream of NKB, although there are very limited data on the relevance of the NKB pathway in normal men or women, and no hierarchical data on this. The studies described in this thesis have investigated the interaction between these neuropeptides in the control of human reproduction in conditions of varying sex-steroid environment, and in states of fast and slow LH secretion (men, menopause, various stages across the menstrual cycle).

Overall hypothesis: Pharmacological blockade of NKB signalling will decrease LH secretion by modulating GnRH/LH pulsatility, indicating the involvement of the NKB pathway in normal human reproductive function. It is also hypothesised that this will not abrogate the stimulatory kisspeptin response, revealing a functional hierarchy whereby NKB signalling is upstream of kisspeptin.

Research strategy: A specific neurokinin-3 receptor antagonist (NK3R antagonist, AZD4901) was administered 40 mg twice daily orally for 7 days with and without kisspeptin-10 (KP-10) challenge. Response of reproductive hormones (serum and urinary where applicable) was measured. LH was sampled every 10 minutes for 8 hours to assess LH pulsatility by blinded deconvolution.

Results:

Role of neurokinin B and kisspeptin in healthy men

Six healthy men underwent LH pulsatility study pre-treatment and on day 7 of NK3R antagonist administration with iv KP-10 bolus (0.3 μg/kg) at 6 hours. NK3R antagonist reduced LH and testosterone secretion, whilst stimulatory LH response to KP-10 was unaffected. LH pulse frequency was unchanged by the NK3R antagonist but basal (nonpulsatile) and pulsatile LH secretion was markedly reduced.

Role of neurokinin B and kisspeptin in postmenopausal women

Eleven postmenopausal women underwent LH pulsatility study pre-treatment and on day 7 of NK3R antagonist administration with iv KP-10 bolus (0.3 µg/kg) at 6 hours. NK3R antagonist decreased LH secretion. Basal (nonpulsatile) LH secretion also fell and while LH pulse frequency did not change in a group as a whole, it did fall in the 8 of 11 postmenopausal womenwith hot flushes. These women reported a reduction in hot flush frequency (3.4±1.2 vs 1.0± 0.6 flushes/day with NK3Ra, p=0.008) and severity whilst on NK3R antagonist. LH response to KP-10 was minimal and unaffected by the NK3R antagonist.

Role of neurokinin B across different phases of menstrual cycle

The effect of NK3R antagonist on ovarian function was compared in early follicular (n=13), late follicular (n=6) and luteal phase (n=6) to no treatment control cycle.

Early follicular: NK3R antagonist was commenced from cycle day 5-6. The diameter of the leading follicle was smaller than in controls at the end of treatment (9.3±0.4 vs 15.1±0.9 mm, p<0.0001). Serum estradiol was also reduced and the endometrium was thinner. Although NK3R antagonist had no effect on LH pulse frequency, basal (nonpulsatile) LH secretion was decreased, suggesting that NKB modulates GnRH secretion. After stopping treatment, follicle development resumed and estradiol secretion increased thereby delaying the LH surge in 11/13 women (LH surge cycle day 22±1 vs 15±1, p=0.0006). The delayed LH surge and ovulation were confirmed by a similarly delayed rise in urinary progesterone and prolonged cycle length. NK3R antagonist did not affect luteal function.

Late follicular: NK3R antagonist was administered from the emergence of a dominant follicle (≥12mm). Whilst there was an LH surge in all treated cycles, estrogen feedback was perturbed by the NK3R antagonist, as there was increased variation in the timing of LH surge compared to control cycle. NK3R antagonist had no effect on the growth of a dominant follicle and luteal function was unaffected.

Luteal: NK3R antagonist was administered from day +2-3 of the disappearance of the dominant follicle. NK3R antagonist reduced the variation in the timing of peak estradiol secretion. Estradiol and progesterone concentrations remained unchanged,

suggesting that luteal function was overall unaffected by this treatment. No difference in mean LH was observed, although LH pulsatility was not assessed.

Role of neurokinin B and kisspeptin in the mid-cycle LH surge

A model of follicular phase (cycle day 9-11) administration of estradiol (200µg/day) to induce LH secretion at 48 hours was used in twenty women, mimicking LH surge. In this model, KP-10 infusion (4µg/kg/hr for 7 hours) enhanced LH secretion, the response of which was directly correlated with estrogen concentration, indicating a role of kisspeptin in estrogen feedback. Pre-treatment with NK3R antagonist decreased LH pulse frequency and whilst the immediate LH response to KP-10 was unaffected, it blunted the duration of this response and abolished the relationship between estradiol and kisspeptin-induced LH secretion.

Conclusions: These data indicate the role of NKB-KP pathway in regulating human reproductive function and that this is via the modulation of pulsatile GnRH secretion. Whilst NKB is predominantly proximal to kisspeptin, the hierarchy is more complex than simply linear in the control of human HPG axis. Manipulation of NKB-KP signalling has therapeutic potential in regulating GnRH/LH secretion in wide range of clinical settings, including contraception, sex-steroid dependent disorders and in the treatment of hot flushes.

Lay Summary

Reproductive function is regulated by the brain hormone called Gonadotropin Releasing Hormone. This in turn stimulates Luteinising Hormone (LH) secretion from the pituitary gland at the base of the brain, which effects how ovaries and testes function in women and men. These hormones are released in bursts (pulses), which are essential for normal reproductive function. It is known that the frequency of burst of those hormones can be regulated by a novel brain hormone called kisspeptin and slowed down by a blocker of another hormone neurokinin B (NKB). However, the interaction between kisspeptin and NKB in the regulation of human reproduction is unknown. Abnormal secretion of those brain hormones might cause low levels of LH and therefore clinical problems such as a delay in puberty, or high levels of LH which may led to a condition such as Polycystic Ovary Syndrome (PCOS), causing infertility. Since the ability to control LH secretion bursts could be used to develop new treatments for various conditions of reproductive function, the aim of this study was to understand how these brain hormones interact and control LH bursts.

The interaction between kisspeptin and NKB was tested in healthy women across varying stages of their reproductive life and in healthy men. During the study, kisspeptin was administered into a vein and NKB blocker was taken as a tablet twice a day for 7 days. LH was measured after taking a blood test and in women with periods, growth of eggs in the ovary was measured by ultrasound imaging.

We found that in women after menopause and in men the blocker of NKB decreased LH levels. The NKB blocker also reduced the male hormone testosterone. Kisspeptin, on the other hand, increased LH levels in men where the frequency of LH bursts is characteristically slow. However this increase in LH levels was minimal in women after menopause (where LH burst frequency is fast). The effect of kisspeptin on LH levels was not affected by the NKB blocker, suggesting that kisspeptin effects on LH are ‘further down the pathway’ of the NKB system. The NKB blocker and kisspeptin also affected various aspects of LH release in bursts in those subjects, indicating that NKB and kisspeptin control reproduction by acting on the main hormone, Gonadotropin Releasing Hormone.

Postmenopausal women taking NKB blocker reported that the frequency of their hot flushes and night-time awakenings were reduced with this treatment, and the severity of their flushes decreased. In this group of women, blockage of NKB reduced the frequency of LH burst and since it is thought that menopausal hot flushes are related to the frequency of LH burst, there is a potential for NKB blockers to be used as new treatments for the management of those flushes.

When administered soon after the period has started in healthy women, NKB blocker was able to delay the growth of the new egg. This subsequently delayed the sharp rise in blood LH levels, which causes that egg to be released. After stopping treatment, normal egg growth and release occurred. When administered at the time when there is a large egg ready to be released, the NKB blocker caused a small delay in the growth of the egg and interfered with the timing of sharp LH rise. This suggests that the communication between the ovary and the brain was interrupted but not completely blocked. When administered soon before the period was due to start the NKB blocker had minimal effect on hormone levels.

Also studied was how the sharp rise in LH that causes an egg to be released from the ovary is regulated by the NKB blocker, or brought on by kisspeptin in healthy women. Kisspeptin increased a rise in LH levels similar to levels seen at the time when egg is being released normally. The blocker of NKB did not abolish this LH rise but made it of a shorter duration. This indicates that NKB regulates how the brain controls egg growth in the ovary, the rise in LH levels and egg release. The blocker of NKB can affect those key events of human reproduction and may be applied to the development of new contraception.

Taken together this data suggest that NKB and kisspeptin are key regulators of normal human reproductive function and that they act by controlling the key brain hormone Gonadotropin Releasing Hormone. The interaction between those hormones is a complex one. The ability to manipulate how NKB and kisspeptin function in the brain by their blockers or stimulators might be able to regulate the frequency of LH burst. This could be subsequently applied to various clinical settings, including delay in puberty, PCOS, menopausal hot flushes, and the development of new contraceptives.

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Abbreviations

ACCORD	Academic and Clinical Central Office for Research and Development
AIC	Akaike information criterion
α-MSH	alpha-melanocyte-stimulating hormone
ApEn	approximate entropy
AR	androgen receptor
ARC	arcuate nucleus
AUC	area under the curve
AVPV	anteroventral periventricular nucleus
BD	twice daily
BIC	Bayesian information criterion
BMI	body mass index
CRF	corticotropin-releasing factor
CV	coefficient of variance
DAG	diacylglycerol
DHT	dihydrotestosterone
DREAM	disorders of reproductive endocrine axis in man
ELISA	enzyme linked immunosorbent assay
ERE	estrogen response element
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
FAS	free alpha subunit
FGF8	fibroblast growth factor 8

FGFR1	fibroblast growth factor receptor
FSH	follicle stimulating hormone
GABA	gamma-aminobutyric acid
GCP	good clinical practice
GFP	green fluorescent protein
Glut2	glutamate transporter-2
GnRH	gonadotropin releasing hormone
GnRH	gonadotropin releasing hormone receptor
GPCR	G-protein coupled receptors
GPR54	G-protein coupled receptor 54 (kisspeptin receptor)
HCG	human chorionic gonadotropin
HFRDIS	hot flush-related daily interference scale
HGNC	Human Genome Organization Gene Nomenclature Committee
HPG	hypothalamic-pituitary-gonadal
HS6ST1	heparin suphate 6-O-sulphotransferase I
ICH GCP	International Conference on Harmonisation Tripartite Guideline for Good Clinical Practice
ICV	intracerebroventricular
IP	intraperitoneal
IP3	inositol triphosphate
IV	intravenous
IVF	in vitro fertilisation
KAL1	cell adhesion molecule anosmin-1
KISS1/Kiss1	kisspeptin gene
KISS1R/Kiss1r	kisspeptin receptor gene

KNDy	Kisspeptin-Neurokinin B-Dynorphin
KP and kp	kisspeptin
LH	luteinizing hormone
LHRH	Luteinizing Hormone Releasing Hormone
LPS	lipopolysaccharide
LRF	Luteinizing Hormone Releasing Factor
MAPK	mitogen-activated protein kinase
MBH	mediobasal hypothalamus
MLE	maximum-likelihood estimation
mRNA	messenger ribonucleic acid
MUA	multi-unit electrophysiological activity
NELF	nasal embryonic LH releasing hormone factor
NHS	National Health Service
NK3R	neurokinin 3 receptor
NK3Ra	neurokinin 3 receptor antagonist
NKB	neurokinin B
Non-CTIMP	none clinical trial of an investigational medicinal product
NPY	neuropeptide Y
OHSS	ovarian hyperstimulation syndrome
P4	progesterone
PBAC	pictorial blood assessment charts
PCOS	polycystic ovary syndrome
PeN	periventricular nucleus
POA	preoptic area
POMC	pro-opiomelanocortin

PR	progesterone receptor
PROK2	G-protein prokineticin 2
PROKR2	G-protein prokineticin 2 receptor
R&D	Research and Development
RP3V	rostral periventricular region of the third ventricle
RT-PCR	reverse transcription polymerase chain reaction
SEMA3A	chemotactic agent semaphorin 3
SEMA3E	sematophorin 3E
SC	subcutaneous
SD	standard deviation
SEM	standard error of the mean
Tac2	neurokinin B gene in rodents homologous to human *TAC3*
TAC3/Tac3	neurokinin B gene
TACR3/Tacr3	neurokinin 3 receptor gene
TSH	thyroid stimulating hormone
USS	ultrasound scan

Chapter 1. Literature Review

1.1 GnRH- the principal regulator of reproduction

Gonadotropin Releasing Hormone (GnRH) is recognised as a key final mediator of endocrine, metabolic and environmental influences on reproductive function conveyed through the central nervous system. Following the pulsatile secretion of GnRH into the hypophyseal portal circulation, it stimulates pulsatile LH and tonic FSH release from the gonadotropes of anterior pituitary, and thus regulates gonadal function and steroidogenesis. This functional unit is known as the hypothalamic-pituitary-gonadal (HPG) axis. Pulsatile secretion of GnRH drives the fundamental events in human reproduction, such as onset of puberty and control of adult fertility, whilst changes in GnRH pulse pattern can lead to reproductive endocrine disorders and sterility.

1.1.1 The discovery of GnRH, its isoforms and receptors

GnRH

The hypothesis that "nerve fibres from the hypothalamus liberate some humoral substance(s) into the capillaries of the primary plexus in the median eminence and that this substance is carried by the portal vessels to excite or inhibit the cells of the pars distalis" dates back to the monograph by Harris in 1955 (Harris. 1955), when mechanisms regulating hormonal secretion from the anterior pituitary were yet unknown. It was not until 1971, when GnRH was first isolated and sequenced from porcine and ovine hypothalami as a 10 amino acid peptide by the two independent groups of the Nobel laureates Andrew V. Shally and Roger Guilleman, respectively (Baba et al. 1971, Schally et al. 1971a). GnRH is a cleavage product of a larger 92 amino acid precursor prepro-GnRH. It was a decapeptide that was initially termed Luteinizing Hormone Releasing Hormone (LHRH) or Luteinizing Hormone Releasing Factor (LRF) (Schally. 2000), but is now known as GnRH to reflect its stimulatory effect on both LH and FSH secretion (Schally et al. 1971b, Conn and Crowley. 1994).

GnRH isoforms

The primary amino acid sequence of mammalian GnRH is not unique but spans for approximately 600 million years of evolution with over 20 isoforms identified in vertebrates (Maggi et al. 2016). GnRHs are named depending on the species in which they were first identified. GnRH was first discovered in mammal and is referred to as GnRH I (Miyamoto et al. 1984, Maggi et al. 2016). GnRH I regulates LH and FSH secretion, and this function will be discussed throughout the thesis. Another isoform was identified from chicken brain and is referred to as GnRH II (Miyamoto et al. 1984, Millar. 2003). GnRH II appears to be a neuromodulator of sexual behaviour (Chen et al. 1998, Millar. 2003). A third isoform was isolated from fish and is termed GnRH III (Sower et al. 1993). It is associated with the control of gametogenesis and sex-steroid production and has anti-proliferative effects, whilst in mammals has insignificant endocrine activity (Deragon and Sower. 1994).

GnRH receptor

The amino acid sequence of receptor for the ligand GnRH I (type I GnRH receptor) was first isolated from the mouse αT3-1 gonadotrope-derived cell line and then cloned from the pituitaries of several species, including humans (Fan et al. 1994). It belongs to the family of G-protein coupled receptors (GPCR) characterised by seven hydrophobic transmembrane domains (Maggi et al. 2016). Upon binding by GnRH, pituitary GnRH receptor couples to the G-protein subunit, activating phospholipase C and yielding secondary intracellular messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG). In turn, IP_3 mediates calcium release, and DAG activates protein kinase C, which amplifies the downstream signalling cascade leading to gonadotropin synthesis (Cheng and Leung. 2005, Maggi et al. 2016). Conversely to episodic stimulation, continuous occupancy of GnRH receptor by its peptide, desensitises gonadotropes, suppressing gonadotropin and gonadal steroid secretion (Conn and Crowley. 1994), the feature of which is extensively applied in clinical management of sex hormone-dependent diseases and in assisted conception. In this thesis, the terms ‘GnRH’ and ‘GnRH receptor’ will be used instead of the GnRH I and its cognate type I GnRH receptor, respectively, when referring to the hypophysiotropic function of the decapeptide.

1.1.2 Neuroanatomy of GnRH network and related developmental disorders

Neurodevelopment

GnRH neurones originate in the nasal region, the medial olfactory placode, and during fetal development migrate along the scaffold of olfactory axons and terminal nerves into the olfactory bulb and to their final destinations within the hypothalamus (Schwanzel-Fukuda and Pfaff. 1989, Wray et al. 1989, Forni and Wray. 2015). The mechanisms regulating GnRH neurone migration involve the timed and coordinated expression of numerous cell adhesion molecules, matrix proteins, guidance cues, neurotransmitters, and growth and transcription factors (Forni and Wray. 2015). Defective GnRH neurone migration with olfactory dysfunction defines the Kallmann syndrome, where hypogonadotropic hypogonadism is associated with anosmia/hyposmia (absent or impaired sense of smell) (Kallmann et al. 1944, Schwanzel-Fukuda et al. 1989). These disorders are recognized as being oligogenic with complex genetic-environmental interactions that account for variable penetrance and interindividual phenotypic variation (Mitchell et al. 2011). Although the familial inheritance has been documented, the majority of cases are sporadic. To date genes that are required for GnRH neurone development, differentiation and function have been implicated in causing hypogonadotropic hypogonadism (Forni and Wray. 2015) and encode for factors including: fibroblast growth factor 8 (*FGF8*) and its receptor (*FGFR1*) (Dode et al. 2003), heparin sulphate 6-O-sulphotransferase I (*HS6ST1*) and nasal embryonic LH releasing hormone factor (*NELF*) (Miura et al. 2004) involved in the embryonic differentiation of GnRH neurones; cell adhesion molecule anosmin-1 (*KAL1*) (Legouis et al. 1991), chemotactic agent semaphorin 3A (*SEMA3A*) (Cariboni et al. 2011), G-protein prokineticin 2 (*PROK2*) and its receptor (*PROKR2*) (Pitteloud et al. 2007) essential for correct migration of GnRH neurones; sematophorin 3E (*SEMA3E*) (Cariboni et al. 2015) regulating the survival of GnRH neurones; neurokinin B (*TAC3*)-neurokinin 3 receptor (*TAC3R*) (Topaloglu et al. 2009) and kisspeptin (*KISS1*) (Topaloglu et al. 2012)-kisspeptin receptor (KISS1R) (de Roux et al. 2003, Seminara et al. 2003) complexes, the upstream mediators of GnRH secretion; and GnRH (*GnRH1*) and the receptor (*GnRHR1*) itself (Bouligand et al. 2009, Chan et al. 2009b).

Distribution of GnRH neurone cell bodies

There are approximately 1000-3000 hypothalamic GnRH neurones, depending on the species studied (Wray and Hoffman. 1986, Maggi et al. 2015). Owning to their olfactory origin, the GnRH cell bodies form a scattered continuum along their migratory pathway, although some species differences exist (Herbison. 2015). In humans, GnRH cell bodies or perikarya are diffusely scattered from the preoptic area (POA) though to the infundibular nucleus (homologue of the arcuate (ARC) nucleus in other species) of the medial basal hypothalamus, whereas in rodents the GnRH cell bodies reside predominantly in the POA (King and Anthony. 1984, Lehman et al. 1986, Schwanzel-Fukuda and Pfaff. 1989, Herbison. 2015).

GnRH neurone projections to the median eminence

GnRH neuronal projections are atypical in that they combine the characteristics of both dendrites and axons, which receive synaptic inputs and propagate action potential, respectively, and hence, have been termed as ‘dendrons’ (Herde et al. 2013). They extend remarkable distances (2-3mm) from the GnRH cell body to the median eminence wrapping around each other and synapsing with adjacent GnRH neurones though numerous spine-like protrusions along the entire length of the dendron (Campbell et al. 2005, Campbell et al. 2009). These interconnections are likely to be functionally important in the synchronization of pulsatile GnRH release (Campbell et al. 2009). GnRH dendrons then break into numerous axons and terminal nerves upon their arrival at the principal site, the external zone of median eminence, where GnRH is secreted into the pituitary portal circulation, allowing delivery of the decapeptide to the gonadotropes of anterior pituitary. At the site of GnRH neurosecretion, GnRH neurones are in close proximity to the hypothalamic network of kisspeptin neurones (Clarkson and Herbison. 2006, Ramaswamy et al. 2008, Hrabovszky et al. 2010), the anatomical distribution of which and their functional interaction with the GnRH system is described below.

1.1.3 GnRH pulsatility

Pulsatile secretion of GnRH is the prerequisite for the acquisition and maintenance of normal adult reproductive function. The pattern of pulsatile GnRH secretion varies across the human lifespan, ranging from high pulsatility during the late stages of gestation and in the first year of life, though to quiescence during childhood, followed by nocturnal reactivation at puberty, culminating in sustained adult pulsatile GnRH secretion. In males, the frequency of GnRH pulses is approximately two hourly, whilst in females it is variable across the different phases of the menstrual cycle with a further surge mode of GnRH secretion mid-cycle (Maeda et al. 2010). Pulse mode refers to the episodic nature of GnRH release into the hypophyseal circulation with undetectable GnRH concentrations in portal blood between those pulses (Maeda et al. 2010). Pulsatile GnRH release drives 'tonic' gonadotropin secretion, necessary for reproductive function in both sexes, such as folliculogenesis, spermatogenesis and the synthesis of gonadal steroids (Plant. 2015a). Surge mode of GnRH release, unique to females, reflects persistence of GnRH in the portal circulation, generating the pre-ovulatory LH surge to induce ovulation (Maeda et al. 2010).

1.1.3.1 Pulsatile GnRH secretion

Pulse mode of GnRH release

The pulsatile nature of GnRH secretion in the primate was initially shown in the rhesus monkey by frequent sampling of the hypothalamic-pituitary stalk portal blood (Carmel et al. 1976). Disconnection of the vasculature links controlling pituitary function precluded those early experiments from simultaneous study of peripheral LH secretion. Although not yet directly demonstrated, the regulation of pulsatile LH secretion by the underlying GnRH pulsatility was postulated and strengthened by the findings that GnRH pulses were in close correlation with previously documented LH pulses in rhesus monkeys (Carmel et al. 1976), that GnRH pulsatility was associated with oscillations in LH levels in the pituitary blood sampled during transsphenoidal surgery in patients with pituitary adenomas (Antunes et al. 1978) and that gonadotropin secretion was abolished by an intravenous administration of antiserum

to GnRH in ovariectomised rhesus monkeys (McCormack et al. 1977). It was not until the early 1980's, when the ability to sample hypothalamo-hypophyseal portal blood and maintain the pituitary function in conscious ewes was achieved, that the synchrony between the pulsatile discharges of GnRH from the hypothalamus and the pulses of LH in the peripheral circulation was demonstrated (Clarke and Cummins. 1982).

The need for intermittent GnRH stimulation of the gonadotropes for sustained LH and FSH secretion was elegantly demonstrated by further work from Knobil's group, where in rhesus monkeys with hypothalamic lesions, abolishing endogenous GnRH and thus gonadotropin release, LH and FSH secretion was reinstated with an intravenous pulsatile GnRH infusion every hour (Belchetz et al. 1978). In contrast, continuous GnRH infusion suppressed this hypophysiotropic response (the process of desensitization), which again recovered when animals were switched from the continuous to pulsatile GnRH replacement (Belchetz et al. 1978). Furthermore, central role of pulsatile GnRH/LH secretion in the regulation of menstrual cycle and response to sex-steroid feedback was demonstrated in rhesus monkeys rendered hypogonadotropic and unresponsive to exogenous estradiol feedback by hypothalamic lesions, where pulsatile GnRH administration re-established normal ovulatory menstrual cycles (Knobil et al. 1980). Gonadal steroids also regulate the frequency of GnRH pulsatility, which is every 2-4 hours in intact rams, increased to a pulse every 70 minutes and every 36 minutes in short-term and long-term castrated rams, respectively (Caraty and Locatelli. 1988).

Surge mode of GnRH release

The surge mode of GnRH release was demonstrated by a massive increase and persistence of GnRH concentrations in the pituitary stalk blood of the proestus rat, correlating with the LH surge and ovulation observed in parallel studies of the same strain of rats (Sarkar et al. 1976). Whether GnRH/LH surge is a summation of high frequency and high amplitude pulses (Rahe et al. 1980, Gallo. 1981, Marut et al. 1981, Norman et al. 1984) or continuous GnRH secretion into the portal circulation remains an ongoing debate (Moenter et al. 1992, Maeda et al. 2010). The latter is favoured by the observation that in sheep, a large amount of GnRH is released prior

the LH surge persisting beyond the end of the LH surge (Moenter et al. 1992). The GnRH surge is triggered by increasing estradiol levels across the late follicular phase of the menstrual cycle in spontaneously ovulating species, or in some species by coitus itself (Plant. 2015a).

Detection of GnRH pulsatility

The half-life of disappearance of exogenous GnRH from peripheral circulation in man is 3.6 minutes with virtually no GnRH present in the blood at the time of maximal gonadotropin stimulation (Arimura et al. 1974). Measurement of GnRH levels outside the portal system therefore cannot accurately reflect its hypothalamic secretion, and in humans, sampling of hypophyseal blood is unsound ethically and practically.

LH pulses in the peripheral circulation remain a widely used and well-validated surrogate of hypophyseal GnRH pulsatility (the long half-life of FSH [274±45 minutes in men] vs LH [approximately 20 minutes] makes FSH an unsuitable marker) (Urban et al. 1991). LH, FSH, Human Chorionic Gonadotropin (HCG) and thyroid-stimulating hormone (TSH) consist of two noncovalently linked polypeptides- α and β subunits. The β subunit is unique to each with hormone specific functional properties, and it is assay to LH β subunit that is used for detection of serum LH levels. The α subunit is conserved in all four hormones and the secretion of the uncombined subunit gives the name of free α subunit (FAS) (Corless et al. 1987). FAS has also been proposed as a marker of GnRH activity due to its short half-live (12-15 minutes) (Whitcomb et al. 1990). However, factors known to modulate gonadotropin secretion, such as starvation, can also alter TSH secretion, precluding FAS correlation with GnRH pulsatility (Samuels and Kramer. 1996). Furthermore, TSH could interact with the human FSH receptor, such as seen in cases of precocious puberty associated with congenital hypothyroidism (Anasti et al. 1995).

1.1.3.2 Pulsatile LH secretion

The pulsatile nature of LH secretion was discovered shortly before the isolation of hypothalamic GnRH. In the course of validation of a radioimmunoassay for serum

LH in rhesus monkeys, Knobil and co-workers reported intermittent rhythmic oscillations in circulating LH concentrations (Dierschke et al. 1970, Knobil. 1981). Further studies in those animals using frequent (10-30min) blood sampling via an indwelling cardiac catheter showed the frequency of those LH pulses to be approximately one hourly (Dierschke et al. 1970). The frequency of LH pulses was higher in ovariectomised rhesus monkeys compared to intact animas with similar findings reproduced in sheep, for the first time suggesting sex-steroid feedback in the regulation of LH pulse frequency (Dierschke et al. 1970, Bolt. 1971). LH secretion in pulses was soon thereafter demonstrated in other mammals, including humans (Katongole et al. 1971, Nankin and Troen. 1971, Butler et al. 1972, Gay and Sheth. 1972, Yen et al. 1972).

Gonadal activity is dependent on the frequency and the amplitude of LH pulses, and furthermore the variation of both parameters in that cycle in females. In males, LH pulse frequency approximately every 2 hours maintains spermatogenesis and testosterone secretion (Nankin and Troen. 1971). In pre-menopausal women, the frequency of LH pulses changes according to the stage of the menstrual cycle, being every 1-2 hours during early follicular phase, early luteal phase and mid-cycle surge while slower every 4 hours in the mid and late luteal phase (Yen et al. 1972). This change in LH pulse frequency is consistent across species (Foster et al. 1975, Baird. 1978, Rahe et al. 1980, Sollenberger et al. 1990). The amplitude of LH pulses also changes from ‘minor oscillations’ in the late follicular phase to higher amplitude during the luteal phase (Yen et al. 1972, Santen and Bardin. 1973). Low LH pulse amplitude in conjunction with rising LH pulse frequency accounts for more than a doubling in LH concentration seen at the mid-cycle LH surge (Yen et al. 1972). Conversely, higher amplitude and lower frequency of LH pulses accounts for decreased LH levels during the luteal phase (Yen et al. 1972, Santen and Bardin. 1973). Pulsatile LH secretion is the major stimulator of ovarian steroidogenesis.

In addition to the frequency of LH pulses regulating ovarian function, the reverse was also shown in human, when in the absence of ovarian steroids in post-menopausal women, LH pulse frequency is as high as during the follicular phase in pre-menopausal women but with high circulating LH concentration (Yen et al.

1972). It is now well accepted that estrogen can be both inhibitory and stimulatory to LH secretion depending on the phase of the menstrual cycle, whilst progesterone is inhibitory. Sex-steroids exert their feedback at the level of both the hypothalamus and the pituitary to modulate pulsatile GnRH/LH secretion. Estrogen acts preliminary to reduce pulse amplitude, while progesterone acts to reduce pulse frequency (Karsch. 1987).

Those early studies above have discovered key paradigms in reproductive biology; the pulsatile nature of LH secretion and the sex-steroid feedback in regulating the frequency and amplitude of the periodic gonadotropin secretion. The novel finding of LH secretion in a pulsatile manner was proposed to be the consequence of then not yet correlated intermittent signals from the central nervous system to the pituitary by an LH releasing factor (Dierschke et al. 1970).

1.1.3.3 Mechanisms of the GnRH pulse generator

To coordinate thousands of neurones to discharge GnRH from the nerve endings into the hypophyseal portal circulation in pulse or surge mode simultaneously, some sort of neural timing mechanism must exist, and is referred to as the GnRH pulse generator or ‘circhoral clock’ (Maeda et al. 2010, Herbison. 2015, Plant. 2015a). Although the importance of the GnRH pulse generator has been recognised for years, its location, and the mechanisms by which GnRH neurones co-ordinate their activity remain incompletely understood (Maeda et al. 2010, Herbison. 2015, Plant. 2015a). Two mechanisms have been proposed for the GnRH pulse-generator; one is that pulsatility is intrinsic to GnRH neurones themselves, and other that a population of non-GnRH neurones within the mediobasal hypothalamus (MBH) target the downstream GnRH neuronal network to drive pulsatile GnRH secretion (Maeda et al. 2010, Herbison. 2015, Plant. 2015a).

GnRH pulse generator: the mediobasal hypothalamus

The localisation of GnRH pulse generator to reside within the MBH comes from the famous ‘Halasz’s knife’ experiments in rats, where deafferentation of the MBH from the rest of the brain did not affect follicle development nor testicular structure, indicating that pulsatile GnRH/LH secretion is preserved (Halasz and Pupp. 1965).

Subsequently, normal LH pulsatility was demonstrated following complete hypothalamic deafferentiation (Blake and Sawyer. 1974, Ohkura et al. 1991), and normal GnRH pulsatility was observed in fetal MBH transplants in adult rat (Ohkura et al. 1992). Recordings of the multi-unit electrophysiological activity (MUA) in the MBH preceding LH pulses further supports the existence of a hypothalamic GnRH pulse generator (Knobil. 1981, Thiery and Pelletier. 1981), although the cells generating those volleys of MUA could not be determined (Maeda et al. 2010). Whilst GnRH neurones are abundant in the primate MBH, they are sparse in the MBH of other species, suggesting MUA volleys may originate outside the GnRH neuronal network (Okamura et al. 2013). It was not until 2003 (de Roux et al. 2003, Seminara et al. 2003), when genetic studies in human populations identified a novel signalling peptide, kisspeptin, which by reciprocally interacting with other hypothalamic neuropeptides neurokinin B and dynorphin located within the MBH, is now considered as a prime candidate for GnRH pulse generation (Okamura et al. 2013). The discovery of kisspeptin and its role in the regulation of pulsatile GnRH secretion will be discussed later in the chapter.

GnRH pulse generator: the autonomicity of GnRH neurones

There is a notion that GnRH pulsatility is intrinsic to the GnRH neurone itself. Immortalised GnRH-secreting GT-1 cells, derived from the mouse hypothalamus, exhibit pulsatile GnRH secretion in the culture medium (Martinez de la Escalera et al. 1992). Similarly, GnRH neurones cultured from the embryonic olfactory placode of the rhesus monkey demonstrated episodic calcium influxes and GnRH pulsatility *in vitro* (Terasawa et al. 1999a, Terasawa et al. 1999b) Furthermore, GnRH neurones themselves exhibit heterogeneous firing patterns, when recorded in GnRH-GFP transgenic rodents, using cell-attached electrodes in acute brain slices and anaesthetised animals (Lee et al. 2010, Constantin et al. 2013).

Synchronisation of GnRH neuronal activity

For GnRH to be released in fixed episodic manner, GnRH neurones require synchronised activation. Axo-dendritic and axo-somatic synapsing between the adjacent GnRH neurones is one possibility by which GnRH neurones achieve co-

ordinated GnRH discharges, although the GnRH network is scattered throughout the hypothalamus, suggesting this would have to operate over significant distances (Witkin and Silverman. 1985, Herbison. 2015). Synchronisation of GnRH pulses may occur at the median eminence, the site where GnRH nerve terminals converge, and is supported by pulsatile GnRH release observed from the isolated rat median eminence in vitro (Rasmussen. 1993). Alternatively, the upstream 'clock' kisspeptin and its interrelated network may affect GnRH cells at the same time, resulting in simultaneous GnRH output (Okamura et al. 2013).

1.1.3.4 Differential secretion of LH and FSH by GnRH pulse frequency

The stimulatory effect of GnRH on gonadotropin secretion is not identical, with FSH being released in constitutive and LH in pulsatile manner (McNeilly et al. 2003). Administration of GnRH antisera in ovariectomised ewes rapidly abolished LH secretion with levels undetectable in 24 hours, whilst FSH secretion fell gradually and remained detectable (Caraty et al. 1984). A change in the frequency of GnRH pulsatile secretion is the key determinant of differential synthesis and secretion of pituitary gonadotropins and the ratio of LH and FSH levels across the different phases of the menstrual cycle. Transcription of LH and FSH β subunits is sensitive to pulsatile GnRH secretion (Haisenleder et al. 1991, Kaiser et al. 1997). Fast GnRH pulse frequency (1 pulse per 60-90 minutes) favours LH release, which is predominant over FSH in the late follicular phase necessary for ovulation, whilst slow GnRH pulsatility (<1 pulse per 2-3 hours) stimulates FSH secretion, which is dominant over LH in the luteal and early follicular stages of the menstrual cycle necessary for follicular development (Wildt et al. 1981). In the course of pubertal acquisition, FSH appears first in the circulation followed by LH, reflecting gradual acceleration of GnRH pulses, further supporting frequency-specific gonadotropin secretion (Burr et al. 1970, Sizonenko et al. 1970). Increasing the frequency of GnRH pulses above one pulse per hour similarly to continuous GnRH administration or an infusion of long-acting GnRH analogue, reduces pituitary responsiveness and extinguishes gonadotropin secretion (Wildt et al. 1981). Although it has no

physiological role, is used pharmacologically in clinical practice (Wildt et al. 1981). In contrast, altering the amplitude of GnRH pulses with constant pulse frequency has minimal regulatory effect on gonadotropic output (Wildt et al. 1981).

1.1.3.5 Relevance of GnRH/LH pulsatility in health and disease

High and low GnRH and thus LH pulse frequency is characteristic of both physiological processes and pathological conditions. Fluctuation in pulsatile GnRH secretion is essential for normal pubertal development and in females, for ovarian function. GnRH/LH pulsatility is low in patients with pubertal delay, hypothalamic amenorrhoea and hypogonadism in diabetes, whereas GnRH/LH pulsatility is enhanced in precocious puberty, polycystic ovary syndrome (PCOS) and menopause. (Berensztein et al. 2006)

Pubertal maturation

Sustained pulsatile GnRH secretion is observed during fetal life and in early infancy and is sufficient to stimulate gonadotropin and sex-steroid secretion, although ovulation and spermatogenesis are not established (Forest et al. 1974, Conte et al. 1980, Waldhauser et al. 1981, Clark et al. 1984). To distinguish this latter phenomenon from true puberty, this phase is referred to as ‘mini-puberty’ (Plant. 2015b). Gonadotropin levels are high during the first 3 months after birth, with LH and FSH decreasing gradually by age 6-9 months in boys, except FSH levels in girls that remain elevated until 3-4 years of age (Kuiri-Hanninen et al. 2014). This gonadotropin surge in associated with gonadal activation. In boys, testosterone peaks at 1-3 months but spermatogenesis is not initiated, as Sertoli cells do not appear to express androgen receptor (Berensztein et al. 2006). In girls, estradiol secretion is more fluctuant, possibly reflecting cyclical development and atrophy of follicles (Kuiri-Hanninen et al. 2014). After this postnatal HPG axis activity, pulsatile GnRH secretion becomes quiescent with stable and slow pulses during childhood, suppressing any activity of the gonads (Plant. 1980, Waldhauser et al. 1981). At the onset of puberty, nocturnal slow GnRH pulses initially stimulate FSH secretion and with acceleration of GnRH pulse frequency LH is secreted (Burr et al. 1970, Sizonenko et al. 1970). The regularity of GnRH and gonadotropin secretion becomes

sustained throughout day and night time, supporting sex hormone production and initiating gametogenesis (Dunkel et al. 1992, Plant. 2015b).

Central mechanisms independent of the gonads appear to be responsible for switching off GnRH pulsatility and maintaining it in low stable state during infancy and then reawakening GnRH pulse generator at puberty (Plant. 2015b, Herbison. 2016b). Agonadal children (Conte et al. 1980) and rhesus monkeys (Plant. 1980) experience the same pre-pubertal suppression of hypothalamic-pituitary-gonadal axis, and adolescent girls with Turner syndrome (45,X) show nocturnal gonadotropin secretion (Boyar et al. 1978).

Premature or delayed reawakening of GnRH pulse generator is the central pathology associated with precocious and delayed puberty, respectively. Mechanisms involved in abnormal activation of the HPG axis include genetic (e.g. mutations in kisspeptin gene, Kallmann syndrome), endocrine (e.g. congenital adrenal hyperplasia), congenital, anatomical (e.g. tumours, trauma), iatrogenic (e.g. irradiation, chemotherapy, gonadectomy) and idiopathic causes (Abreu and Kaiser. 2016).

Maintenance of ovarian cycle

In women, GnRH/LH pulse frequency is lowest in the luteal phase, but increases during follicular and pre-ovulatory phases of the menstrual cycle (Yen et al. 1972, Santen and Bardin. 1973). Distortion of this finely tuned pulsatile GnRH/LH secretion can disrupt the ovarian cycle and give rise to endocrine conditions broadly categorised into those with pathologically low and pathologically high LH pulsatility.

Low LH pulsatility states

Slow GnRH pulsatility characterises hypothalamic amenorrhoea with preferential suppression of LH compared to FSH secretion and subsequently diminished ovarian follicular activity. The frequency of LH pulses in those women is low and comparable to luteal phase in normal women (Santen and Bardin. 1973, Reame et al. 1985). Hyperprolactinaemia inhibits GnRH pulse rhythm resulting in lower LH pulse frequency but higher LH pulse amplitude than that observed in the follicular phase of women with ovulatory cycles (Sauder et al. 1984). Treatment with dopamine

agonists reverses both parameters and restores ovarian function and menses (Sauder et al. 1984). Pulsatile GnRH administration reinstates LH pulsatility in conditions of hypothalamic ovarian failure (Leyendecker et al. 1993). Opioid receptor antagonist (e.g. naltrexone) also increases LH pulse frequency in women with hyperprolactineamia (Cook et al. 1991) and in hypothalamic amenorrhoea, with ovulation and pregnancy documented in the latter (Wildt et al. 1993).

High LH pulsatility states

Compared to the follicular phase of the normal menstrual cycle, women with PCOS have higher serum LH concentrations (Yen et al. 1970). Increased LH pulse frequency and amplitude with little effect on FSH secretion in PCOS presumably reflects a similar pattern of GnRH pulsatility (Santen and Bardin. 1973, Waldstreicher et al. 1988). In addition to high LH pulse frequency, the pattern of LH secretion is irregular as indicated by high Approximate entropy (ApEn) (Veldhuis et al. 2001). Abnormal dynamics of GnRH/LH secretion are one of the pathophysiological processes leading to hyperandrogenism and chronic anovulation characterising this condition. Obese women with PCOS appear to have lower LH pulse amplitude and overall serum LH concentrations compared to lean women with PCOS, reflecting a metabolic component of this heterogeneous disorder (Taylor et al. 1997). Women with PCOS do not exhibit the normal cyclical variation in LH pulse frequency and have constant LH pulses at approximately one hourly. There are currently no licensed therapies aiming at normalisation of LH pulsatility. The first randomised controlled trial to date using a specific neurokinin B receptor antagonist in women with PCOS showed a reduction in LH pulse frequency and subsequently suppressed serum LH and testosterone levels (George et al. 2016). Whether this restores normal ovarian cyclicity remains to be determined.

Menopause and aging

Menopause is associated with increased GnRH and gonadotropin secretion following loss of ovarian activity. Elevated LH pulse frequency in postmenopausal women is similar to that seen in the late follicular phase, with net increase per pulse of LH approaching that during the mid-cycle LH surge (Yen et al. 1972). The pattern of

high LH pulsatility lacks orderliness compared to premenopausal women and is consistent with LH secretion seen in aging men (Pincus et al. 1997, Keenan et al. 2003a). Disordered LH secretion in aging men and women may be associated with diminished negative sex-steroid feedback (Veldhuis et al. 2008).

Diminished GnRH secretion has been observed in early studies in aging men compared to young individuals (Winters and Troen. 1982). Although LH remains responsive to GnRH, the amount of LH secreted per pulse is diminished and hormone release is more disordered in aged men, resulting in declining testosterone production (Mulligan et al. 1999, Keenan and Veldhuis. 2001). A novel analytical method to reconstruct dose-response among reproductive hormones following GnRH receptor antagonist administration suggested testosterone feedback on GnRH/LH secretion to also be impaired in the elderly male, which may contribute to elevated GnRH/LH pulse frequency when compared to younger males (Keenan et al. 2006).

1.1.3.6 Peripheral detection of LH pulsatility

Since GnRH cannot be measured in the peripheral circulation, LH pulse frequency is used as a well-validated marker of GnRH pulsatility. However, there is no generally accepted method to objectively assess LH pulse frequency. The challenge in analysing hormone pulses arises in that hormone secretion patterns are often irregularly spaced in time, nonuniform in size and shape, noisy and superimposed upon a variable baseline with further alterations inflicted by physiological (e.g. age) and pathological (e.g. neuroendocrine tumours, obesity) processes and random effects, such as procedural inconsistencies (missing data, outliers) and measurement variability (Keenan and Veldhuis. 2016).

Subjective pulse identification

Early investigators identified LH pulses by marking its peaks in hormone plots obtained serially over extended intervals (Dierschke et al. 1970, Bolt. 1971). The first two studies of LH pulse frequency in humans defined LH peaks as a series of values consisting of two or more constant or decreasing concentrations followed by two or more higher levels (Nankin and Troen. 1971) in men, and in females, as an increase by 5mIU/ml followed by 5mIU/ml decrease in LH over two sampling points

(Yen et al. 1972). This technique of independent blinded scorers identifying and counting hormone peaks superimposed on marked variability in baseline LH secretion suffers from poor reproducibility (Merriam and Wachter. 1982).

Threshold approaches for pulse detection

Later work has applied some quantitative measures for detecting discrete peaks in hormone concentrations, specifying the threshold increase which is beyond the expected assay variability estimated by the standard deviation (SD) or the coefficient of variation (SD/ mean x 100%). The first of such threshold approaches for pulse analysis was the Santen and Bardin method, for which an increment of greater than 20% from the nadir was accepted as a pulse (Santen and Bardin. 1973). These threshold definitions are however arbitrary and have no physiological significance attached to them. Furthermore, the ability to detect broad LH pulses is limited by this approach. Administration of exogenous LH in gonadotropin-deficient men, has identified the disappearance of LH from the circulation to be in two phases: an initial 'fast half-life' of mean 39 minutes, representing rapid hormone dilution upon secretion into peripheral circulation, and a second 'slow half-life' of mean 121 minutes, indicating metabolic breakdown and excretion (Santen and Bardin. 1973). The half-life of LH secreted in a pulse is inversely related to the peak LH concentration, with high and narrow LH pulses having short half-life and broad small LH pulses having prolonged half-life (Santen and Bardin. 1973). Circadian fluctuations in size (peak) and shape (duration of deviation from the baseline) of LH pulses result in variable basal (nonpulsatile) hormone secretion and increasing hormone half-life, such as in uraemia and hepatic pathology, which elevates interpulse hormone concentration (Veldhuis et al. 1993, Veldhuis et al. 2008). Higher interpulse concentrations diminish the signal-noise ratio, and methods that rely on peak/baseline ratios are therefore less reliable in discriminating pulsatile secretion, especially when pulses are broad. For this reason detection of FSH pulses is more difficult than of LH, as the former has slower half-life (approximately 10.2 hours) and higher basal secretion (Urban et al. 1991, Veldhuis et al. 2008). To take into account assay variability, to more accurately define basal hormone secretion and to detect broad pulses, Baird proposed the LH pulse to be a rise of at least four times

the SD over two consecutive samples, and basal LH secretion to be a mean of two preceding values (Baird. 1978).

The Regional Dual-Threshold method has been proposed to overcome difficulties in pulse detection when hormone concentrations are low (e.g. in puberty) hence increasing the errors of hormone measurement and making fixed threshold methods above invalid (Velduis et al. 1986). Individual coefficients of variation are calculated for all samples using replicate LH assays (e.g. triplicate) at each data point (Velduis et al. 1986). Regional coefficients of variation are then calculated for a particular region of the data, the width of which is operator determined, and threshold multiple of the regional coefficient of variation for the upstroke and downstroke specified to detect pulses (Velduis et al. 1986).

Cluster analysis technique searched for significant increases and decreases in data series (clusters of hormone concentrations) rather than relying on a single data-point to detect pulses (Veldhuis and Johnson. 1986). The significant increase in a cluster of hormone concentrations is compared to the pre peak nadir cluster and a subsequent significant decrease is compared to the post peak nadir cluster, thereby defining a pulse (Veldhuis and Johnson. 1986). An operator determined cluster size of peak and cluster size of nadir (Veldhuis and Johnson. 1986). This technique was advantageous in controlling for random variability by applying multiple-point criteria for peak and nadir detection and allowing a more consistent pulse detection, despite variable baseline hormone concentrations (Veldhuis and Johnson. 1986).

Baseline strategies for pulse detection

Pulse analysis methods employed so called baseline strategies, where computer algorithms identified pulses superimposed on an estimated baseline. Christian and colleagues partitioned blood hormone concentrations into inliers with low hormone levels representing baseline and outliers with abnormally high but random hormone levels representing pulses, which were then identified based on significant skewness of data (Christian et al. 1978). Whilst this approach could not accommodate circadian baseline changes, The Cycle Detector program was proposed to cope with irregular endocrine rhythms and defined a cycle (i.e. pulse) as two increases

separated by a decrease both of which are greater than operator determined threshold value (Clifton and Steiner. 1983). The PULSAR algorithm, on the other hand, calculated smoothened baseline incorporating circadian irregularities in baseline LH secretion and identified pulses based on both their amplitude as well as duration, allowing for detection of both narrow high pulses and broad low pulses (Merriam and Wachter. 1982). The program also factored in assay error and was able to identify clustered peaks (Merriam and Wachter. 1982). Those methods still lacked standardization and required an investigator to set arbitrary artificial assumptions/thresholds.

Deconvolution analysis

Deconvolution is to disentangle or unravel underlying secretion and/or elimination rates from the hormone-concentration profile. It 1) decomposes concentration data into an underlying secretion, which is composed of mass and shape of secretory burst and basal secretion (nonpulsatile or slowly varying); 2) determines elimination of previously secreted hormone and ongoing hormone secretion and elimination; and 3) takes into account random variability (host and assay) (Veldhuis et al. 2008, Keenan and Veldhuis. 2016). A predicted (reconvolution) curve is then constructed by simultaneously estimating pulse amplitude and hormone half-life to fit hormone data (Veldhuis et al. 2008, Keenan and Veldhuis. 2016).

The first one of such approaches was the DETECT computer program. It was able to identify multiple overlapping peaks, evaluate average peak shape and estimate disappearance rate but assumed hormone secretion burst to be instantaneous (zero-duration), which adds ‘noise’ to the basal secretion missing true positive peaks and is unlikely to represent physiological hormone secretion (Oerter et al. 1986).

Deconvolution methods were further defined by the development of so-called blind deconvolution techniques to account for biological variability in half-lives among individuals (Veldhuis et al. 1987a, Keenan et al. 1998). This stemmed from previous deconvolution approaches (DETEC method), which used fixed half-life to calculate of secretion rates (Rebar et al. 1973, Oerter et al. 1986). However, the issue is that hormone half-lives and amplitude concentrations vary depending on physiological

(e.g. age, gender) and pathological (e.g. renal or hepatic impairment) status, and estimate kinetics from one cohort may not be representative of another (Veldhuis et al. 2008). Blind deconvolution estimates secretion rates and hormone half-lives simultaneously, which are unique for each individual and experimental context (Veldhuis et al. 1987a, Keenan et al. 1998, Veldhuis et al. 2008).

The parameters of basal hormone secretion, size (mass) and shape of secretory burst, pulse frequency and hormone half-lives are all interdependent (Veldhuis et al. 2008). Any assumption about these parameters needs careful consideration when placed into a mathematical problem for correct computer-simulated outcome, as repeated analysis of the same data may generate different results. One of these assumptions is the shape or waveform of the hormone secreted, which impacts hormone half-life. An improvement on hormone secretory burst being instantaneous was the application of a Gaussian smoothing function to make hormone bursts symmetrical (Veldhuis et al. 1987a). However, this was arbitrary and risked overestimation of pulse frequency by not capturing delayed hormone half-life (Veldhuis et al. 2008). Direct sampling of pituitary blood every 30 seconds in conscious mares revealed LH secretory burst to be asymmetrical, where LH is secreted rapidly followed by a slow decline to the baseline (Irvine and Alexander. 1993). Accordingly, a flexible waveform gamma model was developed, which combines rapid initial Gaussian (symmetrical) hormone release with slow asymmetrical secretion to the baseline (Keenan et al. 2005, Keenan et al. 2006) hence accounting for variable secretory burst and half-life. In order to predict the true shape of the hormone secretory burst, a recent strategy is to identify independently the onset of hormone burst followed by mathematically verified methods, such as maximum-likelihood estimation (MLE) or Bayesian estimation to predict the secretion, elimination and variability parameters simultaneously on multiple possible sets of pulse-onset times generated (Keenan et al. 2005, Keenan et al. 2006, Liu et al. 2009, Keenan and Veldhuis. 2016). The most probable pulse-time set is then chosen using a statistical model-selection criterion, such as the Akaike information criterion (AIC) or Bayesian information criterion (BIC) (Liu et al. 2009).

Efforts have also been made to better estimate hormone elimination upon its secretion into the bloodstream, which involves initial diffusion (rapid dispersion

within blood), advection (linear flow due to cardiac rhythm) and gradual elimination by metabolism or degradation (Veldhuis et al. 2008). Biexponential formulations using two half-lives more correctly model elimination kinetics compared to monoexponential ones using one half-life (Keenan et al. 2005). The advantage of the biexponential model is that by utilizing slow half-life it determines true baseline, and by using fast half-life it is able to accurately size the secretory burst amplitude, the former of which is otherwise overestimated and the latter underestimated in the model of one half-life thereby attenuating pulse detection (Veldhuis et al. 2008).

Any deconvolution analysis for pulse detection requires validation, which can be achieved by comparing experimentally designed *in vivo* (true) and mathematically generated *in silico* (probable) pulses (Veldhuis et al. 2008, Keenan and Veldhuis. 2016). This is illustrated by further work from the Veldhuis group, attempting to validate their automated deconvolution LH pulse detection procedure (Keenan et al. 2003b, Keenan et al. 2005) using three different experimental contexts (the arcuate multiunit electrical activity and hypothalamo-pituitary portal GnRH pulses in ovariectomised rats and sheep, respectively, and LH infusion in GnRH antagonist treated men) and computer simulation (Liu et al. 2009). Sensitivity and specificity of this deconvolution algorithm were >90% with both experimental and simulated LH data (Liu et al. 2009). Analysis of pulsatile hormone secretion in this thesis is based on this deconvolution approach, which has been kindly performed by Prof Veldhuis, blinded to data, at the Endocrine Research Unit at the Mayo Clinic (Rochester USA).

Approximate entropy as a measure of secretory pattern orderliness

Approximate entropy (ApEn) is not a part of the pulse detection method but is a complimentary statistic measure of the regularity of hormone secretion (Pincus. 1991). ApEn is able to detect subtle changes in the orderliness of subpatterns in sequential measurements over time that is not apparent by alterations in pulse frequency and amplitude (Pincus and Keefe. 1992). It is calculated for any hormone-concentration profile as a nonnegative number, with zero indicating perfect orderliness and larger values corresponding to a more random/irregular hormone secretion pattern (Pincus. 1991). ApEn statistic requires prior specification of r parameter, the threshold value for evaluating reproducibility, and m parameter, the

number of data points (window) over which the recurrence of the pattern is tested. It tests the likelihood that patterns within the threshold will remain within the threshold in the next comparison window (Pincus et al. 1999). Threshold value r is normalised against the standard deviation of each subject hormone-concentration profile and has been predetermined based on data series length. Threshold value r = 20% SD has been validated for data length of ≥ 60 samples with specificity and sensitivity of ApEn of over 90% (Pincus et al. 1999).

1.2 Kisspeptin-the central modulator of GnRH secretion

Since its Nobel Prize winning discovery, hypothalamic GnRH has been recognised as the highest hierarchical centre that initiates and controls reproductive function. The downstream effects of gonadotropin secretion and gonadal activity were attributed to GnRH pulsatility as the primary point that orchestrates the HPG axis. However, functional limitations in the GnRH neuronal network have been identified: in rats, GnRH neurones lack estrogen receptor (ER)-alpha, the principal receptor needed for estrogen feedback (Herbison and Theodosis. 1992). This prompted the search for an intermediary signalling pathway to mediate sex-steroid feedback and hence GnRH secretion. The hypothalamic neuropeptide kisspeptin seems to be providing this missing link as the key upstream regulator of GnRH secretion (Fig. 1.1), following the discovery of its obligate role in human puberty fourteen years ago (de Roux et al. 2003, Seminara et al. 2003). Kisspeptin is now recognised as crucial in the onset of puberty, the regulation of sex hormone-mediated secretion of gonadotropins, and the control of fertility (Pinilla et al. 2012, Skorupskaite et al. 2014).

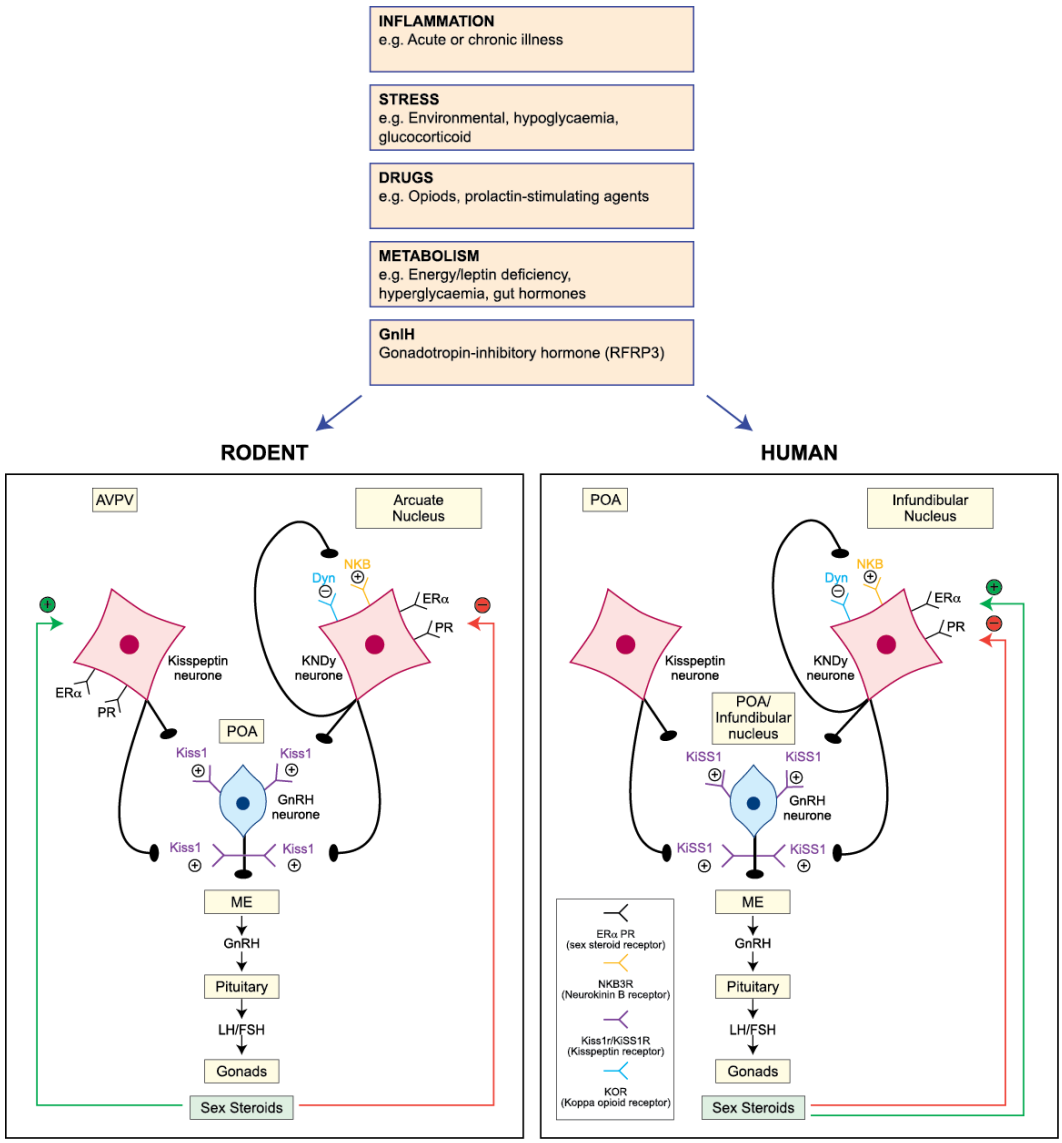


Figure 1.1 The regulation of GnRH secretion by KNDy neurones and other central and peripheral regulators.

GnRH mediates endocrine, metabolic and environmental cues on gonadotropin secretion. Kisspeptin is the upstream modulator of pulsatile GnRH secretion from the median eminence (ME). Kisspeptin neurones reside within the arcuate (rodent)/infundibular (human) nucleus and the rostral area of the hypothalamus, which is more species specific, being located at the pre-optic region (POA) in humans, but at the anteroventral periventricular nucleus (AVPV) in rodents. The arcuate/infundibular kisspeptin neurones co-express neurokinin B and dynorphin (KNDy neurones), which by expressing NK3R and kappa-opioid receptors autosynaptically regulate kisspeptin secretion, with neurokinin B being stimulatory and dynorphin inhibitory. Negative sex-steroid feedback is regulated by KNDy neurones within the arcuate/infundibular nucleus. The anatomical site for positive sex-steroid feedback shows species difference, with the infundibular nucleus in humans but the AVPV nucleus in rodents mediating this function. Adapted from Skorupskaite et al. (2014).

1.2.1 The discovery of kisspeptin gene and receptor

The gene encoding kisspeptins, *KISS1*, was first described in 1996 as a suppressor of metastasis in human malignant melanoma (Lee et al. 1996). Transfection of *KISS1* cDNA into human malignant melanoma cells, C8161, suppressed their ability to metastasise when injected into the tail vein of athymic nude mice (Lee et al. 1996). To acknowledge the discovery of the gene in Hershey (Pennsylvania, USA), it was named after famous chocolate 'Kisses' produced in the town. The SS in *KISS1* also refers to its function as a 'suppressor sequence'. The *KISS1* gene is expressed on the long arm of chromosome 1 (q32) composed of four exons, only the latter two of which are translated (West et al. 1998). It encodes a 145 amino acid peptide, which is cleaved to 54 amino acid sequence with further truncation to 14, 13 10 amino acid length peptides (West et al. 1998). Cleavage products belong to the RF-amide group and share a common C-terminal sequence of Arg–Phe–NH_2 (Kotani et al. 2001).

The orphan G-protein coupled receptor 54 (GPR54) was first described in the rat and then in human brain (Lee et al. 1999, Muir et al. 2001, Ohtaki et al. 2001), with subsequent linkage of kisspeptin as a ligand to its cognate GPR54, now known as KISS1R (Gottsch et al. 2009). *KISS1R* gene is localised to chromosome 19p13.3, where five exons encode 398 amino acid receptor (Muir et al. 2001).

1.2.2 Nomenclature of kisspeptin gene, peptide and its receptor

Since the discovery of kisspeptin-kisspeptin receptor signalling, different terminology has been used throughout the literature to describe the kisspeptin gene, protein and receptor. In this thesis, the nomenclature recommended by Gottsch and colleagues will be used to refer to kisspeptin and its receptor (Gottsch et al. 2009).

1.2.2.1 Nomenclature of the KISS1 gene and mRNA

Lee and colleagues named their newly discovered gene *KiSS-1* (Lee et al. 1996). In the attempt to standardise nomenclature for the mouse, rat and human genome, Gottsch and colleagues have recommended the use of the symbol *KISS1* for the human gene as per Human Genome Organization Gene Nomenclature Committee (HGNC) guidelines and *Kiss1* for non-human kisspeptin gene (Gottsch et al. 2009) (Nomenclature. 2009). Accordingly italicised *KISS1* mRNA and *Kiss1* mRNA are

used to refer to the transcripts of the human and non-human gene, respectively (Gottsch et al. 2009).

1.2.2.2 Nomenclature of the peptide

The 54 amino acid protein was originally named 'metastin' to reflect its antimetastatic properties (Ohtaki et al. 2001). This 54 amino acid metastin and the shorter 14, 13 and 10 amino acid peptides are collectively referred to as kisspeptins given they are translated from the same *KISS1* gene. Non-italicised version of the gene nomenclature would be an alternative to describe the protein product, namely KISS1 for humans and Kiss1 for other species (Gottsch et al. 2009). The name of kisspeptin-54, kisspeptin-14, kisspeptin-13 and kisspeptin-10 are also used when referring to the specific length of the peptide. Others have described kisspeptin based on the numerical sequence of amino acids cleaved the 145 amino acid precursor, with kisspeptin (68-121) referring to kisspeptin-54 and kisspeptin (112-121) indicating kisspeptin-10. Since most authors report the bioactive fragment of kisspeptin used, Gottsch and colleagues have suggested referring to kisspeptin based on their size and abbreviate these to KP for the human and kp in non-human context (Gottsch et al. 2009).

1.2.2.3 Nomenclature of the kisspeptin receptor

The kisspeptin receptor has been referred to as AXOR12, hOT7T175, GPR-54, KISS1, KiSS1 and the metastin receptor. For more consistency, the HGNC denotes *KISS1R* for the human kisspeptin receptor gene, and mouse genome informatics has assigned *Kiss1r* to the once orphaned *GPR-54* (Nomenclature. 2009) Similarly, Gottsch suggested the symbols of *KISS1R* mRNA and *Kiss1r* mRNA for the messenger transcripts of the kisspeptin receptor gene, and KISS1R and Kiss1r for the protein receptors in human and other species respectively (Gottsch et al. 2009).

1.2.3 Discovery of the role of kisspeptin in reproduction

The demonstration of the obligate role of kisspeptin-kisspeptin receptor signalling in human puberty was the finding that firmly established kisspeptin as a crucial regulator of reproductive function. In 2003 two independent groups almost simultaneously identified 'inactivating' point mutations and deletions in *KISS1R* that were associated with delayed pubertal maturation in patients with hypogonadotropic hypogonadism (de Roux et al. 2003, Seminara et al. 2003). Mutations were of both familial and sporadic origins. Genetic findings in humans were reinforced by engineering a *Kiss1r* deficient mice, which displayed a virtually identical phenotype to affected patients with no physiological changes appropriate for the normal pubertal development and low circulating gonadotropin and sex hormone levels (Funes et al. 2003, Seminara et al. 2003). A milder reproductive impact to *Kiss1r* deficient mice was subsequently observed in *Kiss1* knock out mice (d'Anglemont de Tassigny et al. 2007, Lapatto et al. 2007). The pathway downstream of kisspeptin appears unaffected as demonstrated by secretion of gonadotropins in response to GnRH and kisspeptin administration in *Kiss1r* and *Kiss1* mutant mice, respectively (Seminara et al. 2003, d'Anglemont de Tassigny et al. 2007). Conversely 'activating' mutations in the *KISS1* and *KISS1R* genes have been identified in patients with central precocious puberty (Teles et al. 2008, Silveira et al. 2010) (see section 1.2.7 Kisspeptin and puberty for more detail). Since the discovery of the reproductive role of kisspeptin, the field has acquired a wealth of information in understanding of relevant molecular biology, neuroanatomy and physiology, and has progressed to therapeutic manipulation of kisspeptin signalling in the clinical setting.

1.2.4 Kisspeptin receptor signaling

Kisspeptin receptor is a seven transmembrane domain G-protein coupled receptor (Muir et al. 2001). All kisspeptins can bind and activate kisspeptin receptor, but not the galanin or galanin-family peptides, despite the similarity of kisspeptin receptor to the galanin receptor (40% homologous) (Lee et al. 1999). The binding of kisspeptin receptor by its ligand activates phospholipase C (PLC) and recruits secondary intracellular messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG) (Muir et al. 2001, Liu et al. 2008, Constantin et al. 2009). IP_3 mediates a biphasic increase

in calcium from the intracellular stores, with a rapid increase followed by a more sustained second phase (Min et al. 2014). To maintain this second phase and therefore sustain signalling, kisspeptin receptor trafficking involving internalization, recycling and recruitment from an intracellular pool is required (Min et al. 2014). Without receptor trafficking, the kisspeptin receptor undergoes desensitization following an initial acute phase (Min et al. 2014). Depolarisation of GnRH neuronal membrane is achieved by activation of transient receptor potential canonical (TRPC)-like channels and blockade of potassium channels, which are mediated by calcium influx and DAG (Zhang et al. 2008). DAG also activates Protein kinase C (PKC), which is associated with the phosphorylation of mitogen-activated protein kinases (MAPK), such as ERK1/2 and p38, which are also involved in mediating kisspeptin signalling (Kotani et al. 2001).

1.2.5 Neuroanatomy of kisspeptin signalling

The anatomical distribution of the kisspeptin neuronal network and the physiology of kisspeptin-kisspeptin receptor signalling have been studied in a number of species, including humans, with some species variation (Fig. 1.1). The focus in this section will predominantly be on human data, using animal findings in case of significant species variation or where human studies are lacking but there is potential translational application.

1.2.5.1 Distribution of kisspeptin in humans differs from other species

Distribution of kisspeptin cell bodies in the brain

The existence of low levels of *KISS1* mRNA was demonstrated in the human brain tissue by the quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) even before the role of kisspeptins in reproduction was discovered (Kotani et al. 2001, Muir et al. 2001). Identification of the link between kisspeptin gene mutations and delayed puberty (de Roux et al. 2003, Seminara et al. 2003), sparked an interest amongst neuroendocrinologists, and *in situ hybridization* studies on the distribution of kisspeptin neuronal network in the human brain were carried out in autopsy samples from premenopausal and postmenopausal women, localising *KISS1*

expression to the infundibular nucleus (homologous to the arcuate nucleus in other species) only (Rometo et al. 2007). A more recent study by Hrabovszky's lab has created a detailed anatomical map of kisspeptin signalling, localising the majority of kisspeptin cell bodies within the infundibular nucleus, in keeping with earlier human studies, but also identified a second dense population of kisspeptin cells in the rostral pre-optic area (POA) and a small number, probably an extension of the infundibular nucleus, in the infundibular stalk (Hrabovszky et al. 2010).

Whilst localisation of kisspeptin neurones in the infundibular/arcuate nucleus is preserved across the species, the expression of kisspeptin perikarya in the rostral region shows interspecies variation (Clarkson and Herbison. 2006, Pompolo et al. 2006, Ramaswamy et al. 2008, Clarkson et al. 2009b, Hrabovszky et al. 2010). Rodents have a specific rostral kisspeptin neurone population in the anteroventral periventricular nucleus (AVPV) and the periventricular nucleus (PeN), the continuum of this region being referred to as the rostral periventricular region of the third ventricle (RP3V) (Gottsch et al. 2004, Clarkson and Herbison. 2006, Clarkson et al. 2009b). In contrast to rodents, ruminants lack this well-defined RP3V population and have kisspeptin cell bodies scattered throughout the pre-optic region, confirmed by both immunocytochemistry and in situ hybridisation (Franceschini et al. 2006, Pompolo et al. 2006, Smith et al. 2007). No kisspeptin cell bodies were identified in the pre-optic area in castrated male rhesus monkey (Ramaswamy et al. 2008), which compliments the expression of *Kiss1* and *Kiss1r* in those hypothalamic areas detected in previous studies (Shahab et al. 2005, Shibata et al. 2007).

The expression of kisspeptin transcripts and peptides in these areas is under the regulation of sex-steroid feedback. In general, estradiol in females stimulates kisspeptin expression in the AVPV nucleus and suppresses it in the arcuate nucleus (Smith et al. 2005a, Smith et al. 2006b, Herbison. 2008), suggesting that these are the loci of positive and negative feedback regulation respectively. The numbers of kisspeptin cells in the arcuate nucleus of female rodents appear to be two- to fourfold higher than in the RP3V (Goodman and Lehman. 2012). This is consistent with other mammals, where the arcuate kisspeptin population is readily observed and contains more kisspeptin neurones (Smith et al. 2007). Pubertal onset is also associated with

dramatic increase in the number of kisspeptin immunoreactive cell bodies in the AVPV/PeN region of both male and female mice and in *Kiss1* expression in agonadal male and intact female monkeys through pre-pubertal to pubertal development (Han et al. 2005, Shahab et al. 2005, Clarkson et al. 2009a).

Kisspeptin cell bodies are present in the other areas of hypothalamus and outside it: the dorsomedial nucleus in mice and sheep, but not the rat; the ventromedial nucleus of the hypothalamus in the sheep and rat, but not other species; the amygdala in rodents and the bed nucleus of the stria terminalis in rodents and rhesus monkeys (Clarkson and Herbison. 2006, Oakley et al. 2009, Lehman et al. 2010).

Distribution of kisspeptin fibres in the brain

Kisspeptin fibre projections broadly match the distribution of cell bodies across the species, which have been analysed using tract tracing with immunocytochemistry or multi-label immunocytochemistry. Dense network of kisspeptin fibres are found within the two anatomical sites of kisspeptin populations, the infundibular/arcuate nucleus and the region of POA/AVPV, and the site of GnRH neurosection, the median eminence (Clarkson and Herbison. 2006, Ramaswamy et al. 2008, Clarkson et al. 2009b, Hrabovszky et al. 2010). Whilst kisspeptin-immunoreactive fibres are present in other regions of the brain, for example the dorsomedial hypothalamic nucleus in all species, there is also species variation in kisspeptin axonal distribution: fibres are detected in the bed nucleus of the stria terminalis in all species except humans, in the ventromedial nucleus in humans and sheep, but not rodents, and in a variety of rodent forebrain structures, including organum vasculosum of the lamina terminalis and the medial and lateral septum (Clarkson and Herbison. 2006, Pompolo et al. 2006, Oakley et al. 2009, Lehman et al. 2010).

Distribution of kisspeptin receptor in the brain

Unlike the abundance of data on kisspeptin neurone localisation, studies on the neuroanatomical distribution of kisspeptin receptor are rather sparse, those using RT-PCR do not provide information on the localisation of the receptor and lack specificity and validity for quantitative comparison, and those utilising *in situ hybridisation* focus predominantly on the detection of kisspeptin receptor in

particular cell type, especially GnRH neurones (Lehman et al. 2013). Even with these limitations in mind, *Kiss1r* mRNA is consistently localised within the pre-optic area and the mediobasal hypothalamus/arcuate nucleus using both RT-PCR in humans (Kotani et al. 2001, Muir et al. 2001) and monkeys (Shahab et al. 2005, Shibata et al. 2007) and *in situ hybridisation* in rodents (Irwig et al. 2004, Herbison et al. 2010) and sheep (Smith et al. 2009a, Li et al. 2012a). Localisation of *Kiss1r* mRNA in GnRH neurones provides evidence for those cells being direct targets of kisspeptin action. Over 90% of murine GnRH neurones express *Kiss1r* (Han et al. 2005) with frequent co-localisation also observed in rats (Irwig et al. 2004) and non-human primates (Shahab et al. 2005, Shibata et al. 2007). *Kiss1r* mRNA has been detected in the extra-hypothalamic regions of the brain such as hippocampus, amygdala and locus coerules (Lehman et al. 2013). *Kiss1r* is also found in the anterior pituitary in a number of mammals including humans, and in rats has been localised to gonadotropes (Kotani et al. 2001, Richard et al. 2008).

Kisspeptin and GnRH neuronal networks overlap

Kisspeptin axons form dense pericapillary plexuses in the human infundibular stalk, where GnRH is secreted into the hypophyseal portal circulation (Hrabovszky et al. 2010). Axo-somatic, axo-dendritic and axo-axonal contacts between kisspeptin and GnRH axons were also demonstrated in the infundibular stalk, in keeping with data from rodents, sheep and monkeys, where kisspeptin and GnRH neuronal networks are in close proximity (Clarkson and Herbison. 2006, Ramaswamy et al. 2008, Smith et al. 2008a, Hrabovszky et al. 2010, Uenoyama et al. 2011). Furthermore, the appositions between kisspeptin and GnRH fibres have been shown to increase in mouse hypothalamus at the time of puberty (Clarkson and Herbison. 2006). These findings together with the localisation of *Kiss1r* mRNA in GnRH neurones indicate direct involvement of kisspeptin in the neurosecretion of this decapeptide (Irwig et al. 2004, Han et al. 2005, Messager et al. 2005). However, in humans as well as other species studied to date, not all GnRH neurones receive kisspeptin neurone contacts (Clarkson and Herbison. 2006, Ramaswamy et al. 2008, Smith et al. 2008a, Hrabovszky et al. 2010), suggesting that other neuropeptides and neurotransmitters are involved in regulation of GnRH secretion.

1.2.5.2 Kisspeptin neurones co-express other neuropeptides

Kisspeptin cells are a heterogeneous population in that they co-localise with other hypothalamic neuropeptides and neurotransmitters (Fig. 1.1). A subpopulation of kisspeptin neurones co-expressing neurokinin B (NKB) and dynorphin A was first described by Goodman and colleagues in the ovine arcuate nucleus (Goodman et al. 2007). The observation that the distribution and morphology of neurokinin B neurones in the infundibular nucleus in postmenopausal women was similar to that of kisspeptin neurones has suggested the expression of those neuropeptides to localise to the same cell (Rance et al. 1990, Rance and Young. 1991, Rometo et al. 2007). Immunocytochemistry has confirmed the expression of kisspeptin, neurokinin B and dynorphin in the human infundibular nucleus (Rometo et al. 2007, Rance. 2009, Hrabovszky et al. 2010), a phenomenon which is conserved across the species in the analogous arcuate nucleus in rats (Burke et al. 2006) , mouse (Smith et al. 2005a, Navarro et al. 2009) and monkeys (Ramaswamy et al. 2008). Neurokinin B and dynorphin are absent from the kisspeptin population in the pre-optic/RP3V. There is thus a population of infundibular nucleus (human) /arcuate (other species) neurones, which co-express all three neuropeptides, and these, are referred to as KNDy (Kisspeptin-Neurokinin B-Dynorphin) neurones (Cheng et al. 2010).

In rats and sheep KNDy neurones co-localize with the glutamate transporter-2, the ligand of which has been implicated in mediating positive estrogen feedback in the GnRH/LH surge (Pompolo et al. 2003, Ciofi et al. 2006). However, the expression of glutamate receptor in KNDy cells is not yet elucidated (Ciofi et al. 2006, Lehman et al. 2010). Kisspeptin neurones in the RP3V in mouse also co-express tyrosine hydroxylase (the key enzyme in dopamine synthesis), met-enkephalin, GABA and galanin (Ferin et al. 1984, Smith et al. 2006b, Oakley et al. 2009). This differential expression of neurotransmitters suggests that two distinct populations of kisspeptin are phenotypically unique in both their molecular composition and functionality (Oakley et al. 2009).

1.2.5.3 Functional dimorphism of kisspeptin neurones

KNDy neurones form direct contact with both GnRH cell bodies and dendrites in the preoptic area and the mediobasal hypothalamus and project to the median eminence in the sheep, rodents, rhesus monkeys and human (Krajewski et al. 2005, Ciofi et al. 2006, Clarkson and Herbison. 2006, Ramaswamy et al. 2008, Dahl et al. 2009). The arcuate KNDy neuronal network is reciprocally interconnected, allowing for the synergistic neuroactivity of interlinked KNDy cells (Foradori et al. 2002, Burke et al. 2006, Lehman et al. 2010) (Fig.1.1). This is achieved via the neurokinin B receptors and the kappa opioid peptide receptors (the receptor for dynorphin), which are expressed by KNDy cells (Krajewski et al. 2005, Navarro et al. 2009, Herbison et al. 2010), but not the kisspeptin receptor, which predominantly co-localises with GnRH neurones (Irwig et al. 2004, Han et al. 2005, Shahab et al. 2005, Shibata et al. 2007, Herbison et al. 2010). This indicates that by autosynaptic communications between KNDy cells, the stimulatory action of neurokinin B and the inhibitory action of dynorphin achieve coordinate release of kisspeptin, which in turn controls the pulsatile secretion of GnRH and subsequently LH (Navarro et al. 2009).

Kisspeptin-mediated GnRH secretion is sex-steroid dependent as inferred by frequent co-localisation of the nuclear estrogen receptor alpha (ERα), progesterone receptor (PR) and androgen receptor (AR) in kisspeptin as well as neurokinin B and dynorphin expressing cells otherwise absent from GnRH neurones (Ciofi et al. 1994, Goubillon et al. 2000, Foradori et al. 2002, Smith et al. 2005a, Franceschini et al. 2006). There is species specificity in the subpopulation of kisspeptin neurones exerting negative and positive gonadal steroid feedback, described more fully below (1.4). Briefly, in humans, KNDy neurones and possibly additional neuromediators (e.g. glutamate) in the infundibular nucleus alone mediate both negative and positive sex steroid response (Rometo et al. 2007, Oakley et al. 2009), whereas in rodents KNDy neurones in the arcuate nucleus relay negative sex steroid signalling, but it is kisspeptin in the AVPV nucleus that responds to positive sex steroid feedback (Smith et al. 2005a, Smith et al. 2006b, Herbison. 2008). Although humans lack two distinct anatomical sites mediating negative and positive sex-steroid feedback, two separate neuronal pathways are likely to exist to exert different feedback loops.

1.2.5.4 Sexual dimorphism of kisspeptin neurones

There is evidence for sexual dimorphism in kisspeptin pathways across species. Female hypothalami have significantly more kisspeptin fibres in the infundibular nucleus and the rostral periventricular zone compared to men (Hrabovszky et al. 2010). Likewise there is marked sexual dimorphism in the numbers of kisspeptin cell bodies, of which there are more in the female infundibular nucleus but which are absent from the rostral periventricular zone in men (Hrabovszky et al. 2010). Similar sex differences in kisspeptin cell numbers have been reported in the arcuate nucleus of the male and female sheep (Cheng et al. 2010). Female rodents contain more than 10-fold more kisspeptin neurones than males in the RP3V region, a difference that may be explained by pre-ovulatory positive estrogen feedback being unique to the female (Clarkson 2006, Kauffman 2007). Consistent with negative sex-steroid feedback operating in both sexes, sexual dimorphism in kisspeptin expression is not seen in the arcuate nucleus in this species (Clarkson 2006, Kauffman 2007).

1.2.6 Kisspeptin and the regulation of GnRH secretion

Kisspeptin is now recognised as having a central stimulatory role of the hypothalamic-pituitary-gonadal axis in both animal models and humans (Clarke and Dhillo. 2016). Upon binding to kisspeptin receptor on GnRH neurones, kisspeptin mediates pulsatile GnRH release from the median eminence into the portal circulation, which in turn stimulates the synthesis and secretion of LH and FSH from the gonadotropes of the anterior pituitary.

1.2.6.1 Stimulatory effect of kisspeptin on gonadotropin secretion

Kisspeptin effects on LH secretion

Shortly after the discovery of the reproductive role of kisspeptin in genetic studies, the ability of kisspeptin to increase LH secretion was first demonstrated in rodents (Gottsch et al. 2004, Irwig et al. 2004, Matsui et al. 2004, Navarro et al. 2004a, Thompson et al. 2004). Intracerebroventricularly (IVC) administration of 1 nmol of both kp-54 and kp-10 in adult male mice significantly and equipotently increased orbital blood LH levels 30 minutes post kisspeptin injection (Gottsch et al. 2004). A

dose of kp-54 as low as one femtomole was able to elicit a significant LH response (Gottsch et al. 2004). Subsequently, the potency of kisspeptin in stimulating LH secretion was replicated in larger animals, including sheep (Messager et al. 2005, Caraty et al. 2007), gilts (Lents et al. 2008), goats (Hashizume et al. 2010), cows (Ezzat Ahmed et al. 2009) and monkeys (Shahab et al. 2005), where kisspeptin-mediated LH secretion increased in the range of 2 to 25-fold from baseline. Kisspeptin exerts its stimulatory effects on LH secretion via the kisspeptin receptor as *Kiss1r* null mice fails to elicit LH response compared to a robust LH release in wild-type animal with intraperitoneal injections of kisspeptin (Messager et al. 2005).

The Dhillo group was the first to take kisspeptin to man two years after its obligate role in human puberty was described. KP-54 was first administered in healthy men by intravenous infusion (4 pmol/kg/min for 30 minutes followed by half the initial rate for further 60 minutes) and resulted in a robust and dose-dependant increase (from 0.25 pmol/kg/min to 12 pmol/kg/min) in LH, and less marked rises in FSH and testosterone secretion (Dhillo et al. 2005). Subsequently, KP-54 was administered in women by subcutaneous bolus injection at doses ranging from 0.2 to 6.4 nmol/kg causing a dose-dependent increase in serum LH and FSH but not estradiol (Dhillo et al. 2007). The increase in LH was 7-fold more that for FSH, emphasizing the preferential effect of kisspeptin on LH secretion (Dhillo et al. 2007). First in human studies using KP-10 (1.5 μg/kg/hr [1.1 nmol/kg/hr] for 9 h) showed an increase in LH and also the frequency and amplitude of LH pulses in healthy men (George et al. 2011).

Potent LH-releasing effects of kisspeptins have been consistently observed in animals and in humans when kisspeptin is administered (Oakley et al. 2009, Pinilla et al. 2012, Skorupskaite et al. 2014, Clarke and Dhillo. 2016):

1) *by different routes* (central in animals only: intracerebroventricular (icv) or intrahypothalamic; and systemic: intravenous (iv), subcutaneous (sc) or intraperitoneal (ip));

2) *different types of exposure* (single boluses or continuous infusion);

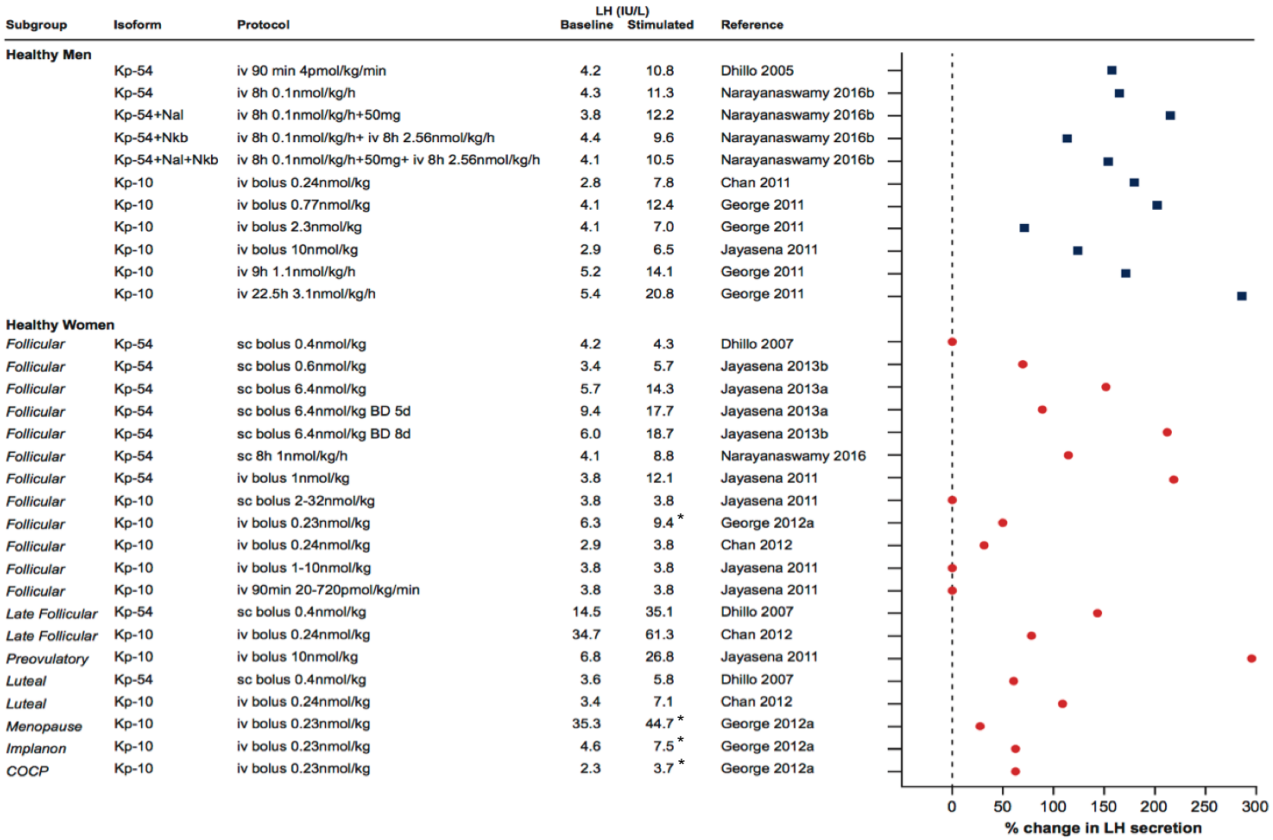
3) *in different isoforms* (kisspeptin-54 and kisspeptin-10);

4) *to males or females*;

5) *in different stages of reproductive function*, including early stages of postnatal development in rat, mouse and monkey (Han et al. 2005, Castellano et al. 2006a, Plant et al. 2006), various phases of the menstrual cycle (Roa et al. 2006, Dhillo et al. 2007, George et al. 2012), lactation (Yamada et al. 2007) and ageing (Neal-Perry et al. 2009, George et al. 2012);

6) *in different disease models and in the IVF setting* (Navarro et al. 2004b, Castellano et al. 2005, Castellano et al. 2006b, Plant et al. 2006, Jayasena et al. 2009, Jayasena et al. 2010, George et al. 2013, Young et al. 2013, Jayasena et al. 2014a, Jayasena et al. 2014b, Abbara et al. 2015).

LH-releasing effects of exogenous kisspeptin administration in human health and disease to date are summarised in figures 1.2 and 1.3.



Subgroup	Isoform	Protocol	LH (IU/L) Baseline	LH (IU/L) Stimulated	Reference
Healthy Men					
	Kp-54	iv 90 min 4pmol/kg/min	4.2	10.8	Dhillo 2005
	Kp-54	iv 8h 0.1nmol/kg/h	4.3	11.3	Narayanaswamy 2016b
	Kp-54+Nal	iv 8h 0.1nmol/kg/h+50mg	3.8	12.2	Narayanaswamy 2016b
	Kp-54+Nkb	iv 8h 0.1nmol/kg/h+ iv 8h 2.56nmol/kg/h	4.4	9.6	Narayanaswamy 2016b
	Kp-54+Nal+Nkb	iv 8h 0.1nmol/kg/h+50mg+ iv 8h 2.56nmol/kg/h	4.1	10.5	Narayanaswamy 2016b
	Kp-10	iv bolus 0.24nmol/kg	2.8	7.8	Chan 2011
	Kp-10	iv bolus 0.77nmol/kg	4.1	12.4	George 2011
	Kp-10	iv bolus 2.3nmol/kg	4.1	7.0	George 2011
	Kp-10	iv bolus 10nmol/kg	2.9	6.5	Jayasena 2011
	Kp-10	iv 9h 1.1nmol/kg/h	5.2	14.1	George 2011
	Kp-10	iv 22.5h 3.1nmol/kg/h	5.4	20.8	George 2011
Healthy Women					
Follicular	Kp-54	sc bolus 0.4nmol/kg	4.2	4.3	Dhillo 2007
Follicular	Kp-54	sc bolus 0.6nmol/kg	3.4	5.7	Jayasena 2013b
Follicular	Kp-54	sc bolus 6.4nmol/kg	5.7	14.3	Jayasena 2013a
Follicular	Kp-54	sc bolus 6.4nmol/kg BD 5d	9.4	17.7	Jayasena 2013a
Follicular	Kp-54	sc bolus 6.4nmol/kg BD 8d	6.0	18.7	Jayasena 2013b
Follicular	Kp-54	sc 8h 1nmol/kg/h	4.1	8.8	Narayanaswamy 2016
Follicular	Kp-54	iv bolus 1nmol/kg	3.8	12.1	Jayasena 2011
Follicular	Kp-10	sc bolus 2-32nmol/kg	3.8	3.8	Jayasena 2011
Follicular	Kp-10	iv bolus 0.23nmol/kg	6.3	9.4 *	George 2012a
Follicular	Kp-10	iv bolus 0.24nmol/kg	2.9	3.8	Chan 2012
Follicular	Kp-10	iv bolus 1-10nmol/kg	3.8	3.8	Jayasena 2011
Follicular	Kp-10	iv 90min 20-720pmol/kg/min	3.8	3.8	Jayasena 2011
Late Follicular	Kp-54	sc bolus 0.4nmol/kg	14.5	35.1	Dhillo 2007
Late Follicular	Kp-10	iv bolus 0.24nmol/kg	34.7	61.3	Chan 2012
Preovulatory	Kp-10	iv bolus 10nmol/kg	6.8	26.8	Jayasena 2011
Luteal	Kp-54	sc bolus 0.4nmol/kg	3.6	5.8	Dhillo 2007
Luteal	Kp-10	iv bolus 0.24nmol/kg	3.4	7.1	Chan 2012
Menopause	Kp-10	iv bolus 0.23nmol/kg	35.3	44.7 *	George 2012a
Implanon	Kp-10	iv bolus 0.23nmol/kg	4.6	7.5 *	George 2012a
COCP	Kp-10	iv bolus 0.23nmol/kg	2.3	3.7 *	George 2012a

Figure 1.2 The stimulatory role of kisspeptin in healthy men and women.

Kisspeptin potently stimulated LH secretion when administered in different isoforms (kisspeptin-54 and kisspeptin-10), by different routes (intravenous and subcutaneous) and protocols (continuous infusion or boluses) in men (filled squares) and across different phases of the menstrual cycle (filled circles). Note that stimulated LH may represent mean LH or peak LH (indicated by asterisk) concentrations depending on data reported in publications. LH levels were obtained from the relevant graphical data, if authors did not state exact values. 0% change in LH secretion indicates no statistically significant change in LH secretion reported whilst other percentage changes in LH secretion are statistically significant. iv, intravenous; sc, subcutaneous; BD, twice daily; COCP, combined oral contraceptive pill.

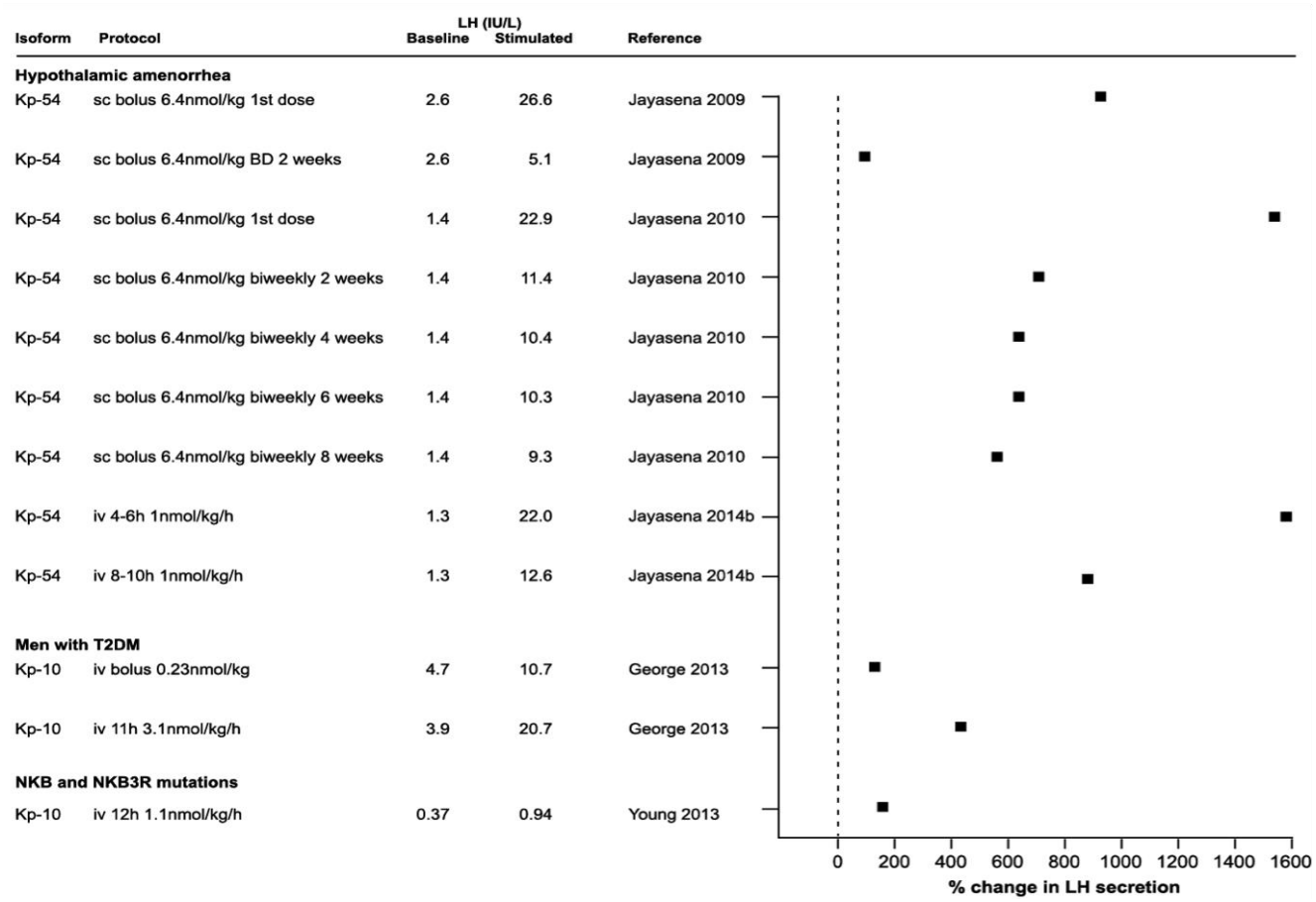


Figure 1.3 The stimulatory role of kisspeptin in human disease models.

The stimulatory effect of kisspeptin on LH secretion in reproductive endocrine conditions characterised by low LH pulsatility. Stimulated LH represents mean LH as reported in publications. LH levels were obtained from the relevant graphical data, if authors did not state exact values. iv, intravenous; sc, subcutaneous; BD, twice daily; T2DM, type 2 diabetes mellitus; NKB, neurokinin B; NK3R, neurokinin 3 receptor.

Kisspeptin effects on FSH secretion

In contrast to LH, the stimulatory effect of kisspeptin on FSH secretion is minimal and more variable in humans (Dhillo et al. 2005, Dhillo et al. 2007, George et al. 2011, Jayasena et al. 2011, Chan et al. 2012, George et al. 2012, Narayanaswamy et al. 2016a), which is in concordance with data in rodents (Thompson et al. 2004, Navarro et al. 2005a). Compared with the rapid (within few minutes) and robust (up to 5-fold) LH response to kisspeptin, FSH release is of slower onset (approximately 30 minutes onwards) and lower magnitude (up to 2-fold) (Thompson et al. 2004, Dhillo et al. 2005, Navarro et al. 2005a, Caraty et al. 2007, Dhillo et al. 2007, George et al. 2011, Jayasena et al. 2011, Chan et al. 2012, George et al. 2012). The stimulatory FSH response to centrally administrated kp-10 in rats is approximately 100-fold less sensitive than for LH with half the maximal response of 4 pmol for LH and 400 pmol for FSH (Navarro et al. 2005a). The preferential secretion of LH to FSH in response to kisspeptin is likely to be related to kisspeptin-mediated increase in GnRH pulse frequency (as inferred by increased LH pulse frequency), with high GnRH pulsatility preferentially stimulating LH synthesis and secretion and low GnRH pulsatility favouring FSH release (McNeilly et al. 2003). Furthermore, FSH secretion is also selectively regulated by gonadal peptides, particular the inhibins (de Kretser et al. 2002), acting at the pituitary.

Gonadotropin response to kisspeptin is modulated by gonadal steroids

When kisspeptin was first administered in healthy women, there was a marked difference in kisspeptin-induced LH and FSH secretion across the phases of the menstrual cycle (Fig. 1.2). Gonadotropin response to exogenous KP-54 (administered at the dose of 0.4 nmol/kg in a subcutaneous bolus) increased over two fold in the pre-ovulatory phase compared to the luteal and follicular phases of the menstrual cycle (Dhillo et al. 2007), suggesting that kisspeptin mediated LH release is dependent on the sex-steroid milieu. Similar enhanced gonadotropin-releasing effect in the pre-ovulatory phase was observed with KP-10 (Jayasena et al. 2011, Chan et al. 2012). In animal models, LH response to kp-10 also depends on the reproductive status with the highest rise in LH observed in the late follicular phase,

which is in concordance with the anatomical studies showing greatest expression of *Kiss1* mRNA at the time of the LH surge in sheep (Smith et al. 2006b, Smith et al. 2009b). Similarly to kisspeptin, intravenous GnRH at various stages of menstrual cycle in women elicited the greatest LH secretion during the pre-ovulatory phase and the smallest during the follicular phase, suggesting that kisspeptin mediates sex-steroid action on gonadotropin secretion via GnRH pathway (Yen et al. 1973, Shaw et al. 1974, Nakano et al. 1975, Jewelewicz et al. 1977, de Kretser et al. 1978).

Estradiol might be an important determinant of LH response to kisspeptin, given that kisspeptin is most effective during the phases of the menstrual cycle when serum estradiol levels are high. Indeed the LH response to subcutaneous KP-54 infusion for 8 hours (0.3 and 1.0 nmol/kg/h) is positively correlated to estradiol levels in the early follicular phase in women with a 100 pmol/l rise in serum estradiol associated with 1.0 IU/l increase in LH response at the higher dose (Narayanaswamy et al. 2016a).

This is consistent with previous *in vitro* and *in vivo* reports where estrogen was shown to be important for kisspeptin-induced GnRH. In GnRH neuronal cell line, estrogen upregulated *Kiss1* gene expression and enhanced kisspeptin-stimulated secretion of GnRH (Li et al. 2007, Novaira et al. 2009, Tonsfeldt et al. 2011). In pubertal monkeys, ovariectomy abolished KP-10 induced GnRH secretion, which was measured directly in the stalk-median eminence by microdialysis, and estrogen replacement reinstated kisspeptin-induced GnRH release (Guerriero et al. 2012).

Somewhat different to the above, sex-steroid deficient postmenopausal women showed a greater AUC LH response to KP-10 than women taking combined estrogen and progesterone contraceptives and those in the early follicular phase of the menstrual cycle (George et al. 2012). It has been proposed that due to high endogenous kisspeptin activity in the follicular phase as a result of loss of negative estrogen feedback, the impact of exogenous kisspeptin is limited (Chan et al. 2012). These complex relationships suggest that, in addition to estrogen and progesterone, other mechanism may regulate kisspeptin sensitivity in different sex-steroid milieu and /or that kisspeptin-GnRH pathways are distinct between women with fluctuating gonadal steroids across the phases of the menstrual cycle, following estrogen-depletion postmenopause and whilst taking exogenous steroids.

Comparison of the stimulatory effects of different kisspeptin isoforms

Whilst the potency of kisspeptin to induce gonadotropin secretion has been evaluated in many different species using various isoforms, routes, regimens and doses, there are limited number of studies comparing kisspeptin effects using the same experimental conditions. Shorter and longer kisspeptin peptides may be distinct pharmacodynamically and pharmacokinetically. A recent study has for the first time compared the effects of KP-10, KP-54 and GnRH on the secretion of gonadotropins when administered at equimolar doses (0.1, 0.3 and 1.0 nmol/kg/h) by an intravenous infusion for 3 hours in healthy men (Jayasena et al. 2015a). Dose-dependent increase in both LH and FSH secretion was observed during the infusion of GnRH and to lesser extent during KP-10 and KP-54 infusions, with peak hormone concentrations achieved at the 0.3 nmol/kg/h dose for LH and 1.0 nmol/kg/h dose for FSH (Jayasena et al. 2015a). At the doses achieving maximal stimulation, AUC serum LH and FSH were 3-fold higher during GnRH infusion compared with KP-10 administration and 2-fold higher than that with KP-54 (Jayasena et al. 2015a). Similarly, in male rats, intravenous bolus injection of GnRH (32.5 nmol/kg) evoked greater LH response than that induced by kp-10 (30 nmol/kg) administered repetitively at four boluses every 75 minutes (Tovar et al. 2006). GnRH potency over kisspeptin in stimulating gonadotropin secretion might be explained by suboptimal GnRH-releasing effect of kisspeptin since not all GnRH neurones are in apposition with kisspeptin neurones or express kisspeptin receptors (Irwig et al. 2004, Clarkson and Herbison. 2006, Ramaswamy et al. 2008, Smith et al. 2008a, Hrabovszky et al. 2010). This suggests that kisspeptin independent pathways may also regulate GnRH secretion (Chan et al. 2009a). Although GnRH secretory nerve terminal are outside the complete blood-brain-barrier in the median eminence (d'Anglemont de Tassigny et al. 2010), not all kisspeptin may penetrate into the hypothalamus (Jayasena et al. 2015a).

Rodent studies suggest that full-length kisspeptin is more effective in stimulating gonadotropin secretion than shorter isoforms (Tovar et al. 2006, Pheng et al. 2009). When 3.0 nmol/kg of kp-52 (analogous to KP-54 in humans) and kp-10 was injected intravenously in male rats, a greater magnitude of LH secretion was achieved with the longer fragment (Tovar et al. 2006). Similarly, subcutaneous bolus injection of

kp-54 at 1 and 50 nmol significantly increased LH secretion in male rats when compared with kp-10 and kp-14 at equivalent doses (Thompson et al. 2006). However, a more detailed study compared the potency of intraperitoneal injection of mouse kp-10 and -52 and human KP-10 and -54 (at 0.1, 0.3, 1, 3, 10 and 30 nmol/kg for each peptide) on testosterone secretion in adult male mice, showing a dose-dependent increase in testosterone concentrations with the same efficacy across the peptides (Mikkelsen et al. 2009). LH levels were not analysed in the study.

Interspecies variability may account for distinct potency of various kisspeptin fragments as well as differences in the dose and mode of administration. Whilst central (ICV) and peripheral (iv and ip) administration of kp-10 and kp-52 in male rats elicited comparable effects on LH secretion, that of peripheral administration was less protracted lasting less than 3 hours (Navarro et al. 2005b, Rance. 2009). Shorter stimulatory action of peripherally delivered kp-10 and kp-54 was also observed in sheep and monkeys (Shahab et al. 2005, Caraty et al. 2007) with generally longer response to intravenous injection than other routes of peripheral administration (Navarro et al. 2005b, Tovar et al. 2006, Caraty et al. 2007).

KP-54 elicits a marked stimulatory effect on gonadotropin secretion when administered intravenously or subcutaneously in healthy volunteers and in women with hypothalamic amenorrhoea (Dhillo et al. 2005, Dhillo et al. 2007, Jayasena et al. 2009, Jayasena et al. 2014b, Jayasena et al. 2015a, Narayanaswamy et al. 2016c). In contrast, difference in KP-10's ability to stimulate gonadotropin secretion has been observed with different routes of peripheral administration. Whilst intravenous KP-10 infusion stimulates LH secretion in men, women and patients with diabetes mellitus and with mutations in the neurokinin B pathway (Chan et al. 2011, George et al. 2011, Jayasena et al. 2011, Chan et al. 2012, George et al. 2012, George et al. 2013, Young et al. 2013), subcutaneous injection of KP-10 failed to stimulate gonadotropin secretion in the early follicular phase in healthy women (Jayasena et al. 2011), although subcutaneous administration of the shorter length peptide has not been evaluated in men, in other phases of menstrual cycle in women and in hypothalamic amenorrhoea.

Differences in KP-54 and KP-10 effectiveness might be explained by a much shorter plasma half-life of KP-10 of 4 min (Jayasena et al. 2011) compared to 27.6±1.1 min for KP-54 (Dhillo et al. 2005). Furthermore, sexual dimorphism in the responsiveness to KP-54 and KP-10 has been demonstrated with men generally showing greater stimulatory effect to both kisspeptin isoforms (Jayasena et al. 2011), discussed in more detail in section 1.2.6.7: Sexual dimorphism in response to kisspeptin. Comparison of the stimulatory action of various kisspeptin lengths is further complicated in that kisspeptin-induced gonadotropin secretion is dependent on gonadal steroid milieu (Dhillo et al. 2007, Smith et al. 2009b, Jayasena et al. 2011, Chan et al. 2012, George et al. 2012).

1.2.6.2 Direct and indirect actions of kisspeptin on GnRH neurones

Direct action of kisspeptin on GnRH secretion

Kisspeptin mediates its stimulatory role on gonadotropin secretion by acting directly on the hypothalamic GnRH pathway, evidence for which is mainly inferred from animal studies. In addition to anatomical data, the physiological role of kisspeptin in the regulation of GnRH secretion has been demonstrated *in vitro* and *in vivo*. Kisspeptin causes depolarisation of and increases in firing rate of GnRH neurones (Han et al. 2005, Zhang et al. 2008, Constantin et al. 2013); kisspeptin-induced GnRH secretion has been observed in hypothalamic explants and the median eminence (Thompson et al. 2004, Tovar et al. 2006); c-Fos immunoreactivity (a marker of neuronal activity) (Matsui et al. 2004, Han et al. 2005) and the expression of *GnRH* mRNA is upregulated within the cell bodies of GnRH neurones following the kisspeptin exposure (Novaira et al. 2009, Oakley et al. 2009).

Temporal correlation between kisspeptin administration and GnRH secretion was first shown in sheep, where central infusion of KP-10 for 4 hours caused a parallel and dramatic increase in the cerebrospinal fluid GnRH content and peripheral LH and FSH concentrations (Messager et al. 2005). In a primate model, central infusion of KP-10 stimulated GnRH release in ovary-intact prepuberal and pubertal monkeys, when measured directly in the stalk-median eminence by microdialysis (Guerriero et al. 2012). In human setting, direct measurement of kisspeptin-induced GnRH

secretion is precluded ethically and to some extent mechanistically, and therefore GnRH-releasing effects in response to kisspeptin is inferred from indirect methods, such as assessment of LH pulsatility (1.1.3.2 Pulsatile GnRH secretion).

Studies using kisspeptin antagonist further support the notion that kisspeptin mediates its action predominantly via the release of hypothalamic GnRH (Millar et al. 2010). Infusion of kisspeptin antagonist into the median eminence of prepubertal and pubertal female rhesus monkeys suppressed both GnRH secretion and pulse frequency, suggesting the role of kisspeptin in GnRH pulsatility (Roseweir et al. 2009, Guerriero et al. 2012). Indirect evidence for the role of kisspeptin in regulating pulsatile GnRH secretion is further demonstrated by reduced LH pulse frequency with kisspeptin antagonist in the female rat and sheep (Li et al. 2009, Roseweir et al. 2009, Smith et al. 2011). Furthermore, reduced LH pulsatility was achieved with kisspeptin antagonist infusion at the arcuate nucleus but not the pre-optic area in rat, suggesting that kisspeptin modulates pulsatile GnRH secretion at the arcuate nucleus, thought to be the site of the GnRH pulse generator (Plant et al. 1978a). In contrast to kisspeptin, exogenous GnRH elicited a prompt LH release in *Kiss1r* knockout mice, suggesting that pituitary responsiveness is preserved and that and kisspeptin signalling to be proximal to GnRH (Seminara et al. 2003).

The action of kisspeptin to be operating upstream of GnRH is elegantly demonstrated by abrogation of kisspeptin-induced LH secretion in response to GnRH antagonist. Rodents and agonadal male monkeys pre-treated with a potent GnRH receptor antagonist, acycline, failed to mount gonadotropin rise following an otherwise robust response to kp-54 and kp-10 administration, respectively (Gottsch et al. 2004, Matsui et al. 2004, Shahab et al. 2005). Passive immunisation of ovariectomised ewes with GnRH antiserum achieved consistent results with kp-10 failing to induce LH pulse and increase serum LH concentrations (Arreguin-Arevalo et al. 2007). Although there are no human studies administering kisspeptin antagonist or GnRH antagonist followed by kisspeptin, the direct action of kisspeptin on GnRH neurons is inferred from the consistent findings in other species, including nonhuman primates.

Indirect action of kisspeptin on GnRH secretion

In addition to direct stimulatory role of kisspeptin on GnRH neurones, kisspeptin appears to regulate GnRH secretion though its action on other intermediary signalling pathways, such as GABAergic (excitatory and inhibitory) and glutamatergic (excitatory) cells. In the female mice brain slices, blockade of γ-animobutyric acid $(GABA)_A$ receptor (excitatory) and glutamate receptor reduced GnRH neurone firing response to kisspeptin-10 (Pielecka-Fortuna et al. 2008). This is consistent with rodent pre-optic kisspeptin neurones co-expressing GABA and galanin (Ferin et al. 1984, Smith et al. 2006b) suggesting the role of those neurotransmitters in mediating indirect actions of kisspeptin on GnRH secretion.

Kisspeptin-independent GnRH secretion

Kisspeptin-independent GnRH secretion is supported by neuroanatomical studies, showing that not all GnRH neurones express the kisspeptin receptor (Irwig et al. 2004) and that the contacts between the GnRH and kisspeptin neuronal networks actually appear infrequent (Clarkson and Herbison. 2006, Ramaswamy et al. 2008, Smith et al. 2008a, Hrabovszky et al. 2010). Although humans with mutations in kisspeptin pathway show failure in pubertal development and infertility (de Roux et al. 2003, Seminara et al. 2003, Topaloglu et al. 2012), *Kiss1* and *Kiss1R* knock-out mice exhibit partial sexual maturation with vaginal estrous phase in females and spermatogenesis in males, suggesting some GnRH activity (Chan et al. 2009a). To confirm that the partial sexual development in those mice is due GnRH secretion, administration of a GnRH antagonist, acycline, resulted in disrupted vaginal estrous, lower uterine and testicular weights, impaired spermatogenesis and low gonadotropin secretion (Chan et al. 2009a). These animals, however, do not undergo ovulation and are infertile, suggesting insufficient GnRH secretion (Lapatto et al. 2007).

Studies using kisspeptin antagonists further suggest that pathways other than kisspeptin signaling are involved in the regulation of GnRH secretion (Millar et al. 2010). Kisspeptin antagonist did not suppress basal LH secretion in gonadal intact and castrate rodents and sheep (Roseweir et al. 2009), when administered directly into the rat arcuate nucleus (Li et al. 2009) and in the pre-ovulatory LH surge studies

in rats (Pineda et al. 2010). Furthermore, basal GnRH secretion was also unaffected by the kisspeptin antagonist in pubertal female monkeys, although GnRH pulse frequency was reduced (Roseweir et al. 2009) as was LH pulsatility in female rats (Li et al. 2009). These findings indicate that basal LH secretion appears to be mediated by kisspeptin-independent pathways on GnRH neurones.

Kisspeptin-independent GnRH secretion could be intrinsic to GnRH neurones themselves. Autonomous GnRH release in synchronous pulses has been observed from GnRH cell lines (Martinez de la Escalera et al. 1992). Ongoing GnRH secretion in the presence of defective kisspeptin signalling may reflect heterogeneity between GnRH neurones. The existence of two physiologically distinct subpopulations of GnRH neurones have been proposed in the prepubertal and pubertal mice; one that co-localises with *Kiss1R* mRNA and VGlut2 and is highly sensitive to kisspeptin but does not respond to glutamate receptor agonist; a second that is insensitive to kisspeptin but is activated by the glutamate receptor agonist (Dumalska et al. 2008). Neurotransmitters, other than kisspeptin, such GABA, galanin, glutamate, norepinephrine, neuropeptide Y and RF-amide-related peptides, have been implicated in modulating GnRH secretion, although evidence for their critical role is lacking for some (Herbison. 2015). Type I GnRH receptor transcripts have been co-localised to the mice GnRH neurones, although the direct effects of GnRH on its neurone excitability remain unclear (Xu et al. 2004, Todman et al. 2005). The action of those neuromodulators upon GnRH neurones is reviewed in detail elsewhere (Herbison. 2015).

1.2.6.3 Pituitary effects

The direct action of kisspeptin at the pituitary gonadotrope remains debatable. The expression of both *Kiss1* and *Kiss1r* genes have been detected in gonadotropes in a number of species, including humans, baboons, sheep, pigs and rats (Kotani et al. 2001, Gutierrez-Pascual et al. 2007, Li et al. 2008, Richard et al. 2008, Smith et al. 2008b). Kisspeptin is able to induce gonadotropin release from pituitary explants and cells *in vitro*, although its stimulatory effect on LH is modest compared to GnRH despite the use of kisspeptin concentrations well beyond those considered

physiological (Gutierrez-Pascual et al. 2007, Suzuki et al. 2008). Furthermore, kisspeptin has been detected (although in low levels) in the hypophyseal portal circulation in the sheep, suggesting that hypothalamic kisspeptin is transported in the portal blood to exert its action directly on the gonadotropes (Smith et al. 2008b). However, no fluctuations in kisspeptin levels have been observed in the portal blood to reflect changes in sex-steroid environment across the reproductive span (Smith et al. 2008b). In the hypothalamic-pituitary disconnected sheep, kisspeptin failed to induce LH secretion, suggesting that kisspeptin acts upstream of the pituitary (Smith et al. 2008b). This is further supported by the failure of kisspeptin to stimulate LH release in GnRH antagonist pre-treated monkeys (Gottsch et al. 2004, Irwig et al. 2004). Whilst there is evidence to suggest direct action of kisspeptin on gonadotropes, it is clear that kisspeptin predominantly exerts its stimulatory role on LH and FSH secretion indirectly through GnRH (Gottsch et al. 2004, Irwig et al. 2004, Smith et al. 2008b).

1.2.6.4 Kisspeptin as GnRH pulse generator

There is growing evidence to suggest that a population of kisspeptin neurones located in the hypothalamic arcuate nucleus constitutes the GnRH pulse-generator, which coordinates synchronised and episodic GnRH discharges from its neuronal terminals. In addition to kisspeptin making close associations with GnRH neurones at the median eminence and being a potent GnRH and LH secretagogue, there are anatomical and functional data in animals and humans supporting this notion.

The arcuate kisspeptin neurones in GnRH pulse generating activity

Although the MBH was argued to be the site for a GnRH pulse generating mechanism as early as 1980's (based on the MUA recordings) (Knobil. 1981), kisspeptin neurones as the source of the GnRH pulse generator was not proposed until thirty years later, when in goats, rhythmical MUA volleys were recorded at close vicinity of the arcuate (part of the MBH) kisspeptin cells and in association with LH pulses (Ohkura et al. 2009, Wakabayashi et al. 2010). Importantly, pulsatile kisspeptin-54 release in association with GnRH pulses was detected within the MBH in monkeys (Keen et al. 2008). Whilst kisspeptin antagonists administered centrally

did not abrogate basal GnRH and LH secretion (Li et al. 2009, Roseweir et al. 2009, Pineda et al. 2010), the frequency of GnRH and LH pulsatility was suppressed (Li et al. 2009, Roseweir et al. 2009). It is plausible that kisspeptin is primarily involved in the regulation of pulsatile GnRH and subsequently LH release rather than the maintenance of basal gonadotropin secretion. This is consistent with the abolishment of LH pulsatility following direct kisspeptin antagonist infusion into the arcuate nucleus (but not the pre-optic area) in rats and sheep (Li et al. 2009, Goodman et al. 2013), the proposed site of the GnRH pulse generator (Plant et al. 1978b). Furthermore, female rats bearing kisspeptin knockdown in the arcuate nucleus had suppressed LH pulsatility, indicating the role for kisspeptin within this area for pulse generation (Beale et al. 2014).

In the studies using chronic subcutaneous kisspeptin administration to block kisspeptin-kisspeptin receptor signaling, MUA volleys remained unchanged despite complete suppression of LH secretion (Yamamura et al. 2014). Similarly, peripheral injection of kp-10 elicited LH response but did increase the amplitude or frequency of MUA recording in rats and goats (Kinsey-Jones et al. 2008, Ohkura et al. 2009), suggesting other cells/mechanisms than kisspeptin to be the GnRH pulse generator. However, kisspeptin neurones are deficient of kisspeptin receptors (Herbison et al. 2010, Smith et al. 2011) and it is feasible that peripherally administrated kisspeptin analogues in the studies above did not affect the arcuate GnRH-pulse generator, but rather mediated their effects on the downstream kisspeptin receptor-expressing GnRH dendrons in the median eminence, which is outside the blood-brain-barrier. This may also be the case with antagonistic studies, where the precise location of the kisspeptin antagonism is unclear. Okamura and colleagues have debated whether kisspeptin is the intermediary mechanism which transmits the action potential from the pulse generator to GnRH neurones and mediates GnRH secretion at its terminals upon the entrance to the median eminence (Okamura et al. 2013). This is supported by the stimulatory role of kisspeptin and the inhibitory effect of kisspeptin antagonist on GnRH secretion from the median eminence *in vitro* and *in vivo* (d'Anglemont de Tassigny et al. 2008, Keen et al. 2008).

Effects of kisspeptin on GnRH pulse generator in humans

In humans, studies aiming to delineate the role of kisspeptin in the control of GnRH pulse generator are more challenging and hence sparse. It has however been suggested that in men, kisspeptin can reset the hypothalamic clock that drives pulsatile GnRH secretion (Chan et al. 2011). Acute injection of KP-10 induced an immediate LH pulse and delayed the next endogenous LH pulse by approximately 2 hours (Chan et al. 2011), the interval that would be observed between two consecutive endogenous LH pulses (Santen and Bardin. 1973). A refractory delay following kisspeptin-mediated LH pulse is unlikely since in some men an endogenous LH pulse occurred close to kisspeptin administration (Chan et al. 2011). The morphology of kisspeptin-induced LH pulses in healthy men (Chan et al. 2011) and GnRH-induced LH pulses in those with isolated GnRH deficiency (Pralong et al. 1996) are similar, suggesting that kisspeptin generates LH pulse via a pulse of GnRH. Furthermore, KP-10 appears to induce prolonged GnRH secretion. In men, the morphology of kisspeptin-induced LH pulse was reproduced by a 17-minute infusion of GnRH (Chan et al. 2011). This is in concordance with the duration of kisspeptin-induced GnRH neurone depolarization *ex vivo* (Han et al. 2005, Pielecka-Fortuna et al. 2008, Zhang et al. 2008).

The GnRH clock resetting potential of kisspeptin shows sexual dimorphism as the same kisspeptin dosing protocol to that in men failed to achieve this in women across the different phases of the menstrual cycle (Chan et al. 2012). The authors suggested that the GnRH pulse generator in men operates differently to women and that it is the change in the sex steroid milieu across the menstrual cycle in the women that might be responsible for this discrepancy (Chan et al. 2012). This is consistent with early studies suggesting that in men, GnRH pulse generation is a renewal process adjustable to the last secretory phase, but may not operate in women in the luteal phase of the menstrual cycle (Butler et al. 1986, Santoro et al. 1988). Optogenetic stimulation of the arcuate kisspeptin neurones in female mice required higher frequency of activation to elicit LH pulses, which were still below the amplitude than that observed in males (Han et al. 2015). Sexual dimorphism in spontaneous electrical activity of the arcuate kisspeptin neurones has also been demonstrated in

mice (de Croft et al. 2012). The marked sex difference in the anatomy of the kisspeptin neuronal network in animals and humans may underlie gender specific mechanisms in generating GnRH pulses and may even determine changes in the frequency of GnRH secretion across the menstrual cycle.

1.2.6.5 Effects of kisspeptin on LH pulsatility

As GnRH secretion is pulsatile and kisspeptin is proposed to be an upstream GnRH pulse generator, the effect of kisspeptin on different aspects of pulsatility (as inferred by LH pulsatility) has been investigated. The stimulatory role of kisspeptin on pulsatile LH secretion has been demonstrated in animals and humans, data for the former of which is mainly inferred by studies using kisspeptin antagonism (see section 1.2.6.2 Direct and indirect actions of kisspeptin on GnRH neurones; 1.2.6.4 Kisspeptin as GnRH pulse generator) whilst in humans using exogenous kisspeptin peptide itself (Pinilla et al. 2012, Skorupskaite et al. 2014, Clarke and Dhillo. 2016).

The effect of kisspeptin on LH pulsatility was first demonstrated in humans following an intravenous infusion of KP-10 for 9 hours (1.5 μg/kg/hr [1.1 nmol/kg/hr]), which in healthy men not only increased LH and testosterone secretion but has also enhanced the frequency and amplitude of LH pulses (George et al. 2011). This is in concordance with animal data, where kisspeptin antagonism potently suppressed the frequency of GnRH pulses in monkeys (Roseweir et al. 2009) and LH pulses in rats (Li et al. 2009, Pineda et al. 2010) and sheep (Roseweir et al. 2009, Smith et al. 2011, Goodman et al. 2013), indicating that kisspeptin actions are via GnRH secretion and that kisspeptin modulates GnRH pulsatility.

Interestingly, data from other investigators have not replicated increased LH pulsatility in response to an acute bolus of KP-10 injection in healthy male and female subjects (Chan et al. 2011, Chan et al. 2012), suggesting that a continuous or repeated administration of kisspeptin is needed to modulate GnRH and hence LH pulsatility given short half-life of KP-10 (Jayasena et al. 2011). In contrast, a single injection of KP-54 (0.3 nmol/kg [1.76 μg/kg] and 0.6 nmol/kg [3.55 μg/kg] subcutaneous) temporarily increased LH pulsatility in healthy women (Jayasena et al. 2013b). Although kisspeptin-54 remained detectable in serum for the duration of

the study, 4-hour post injection blood sampling period may not be sufficient to determine all the characteristics of LH pulsatility. Prolonged exposure to KP-54 had no effect on the frequency and amplitude of LH pulses when administered subcutaneously as an infusion for 8 hours (0.3 nmol/kg/hr and 1.0 nmol/kg/hr) (Narayanaswamy et al. 2016a) or as twice daily injection for a week in healthy women during the follicular phase (Jayasena et al. 2013a). Lack of kisspeptin response might reflect relatively low estradiol levels in the follicular phase given that kisspeptin-stimulated gonadotropin secretion is positively correlated to estradiol levels (Narayanaswamy et al. 2016a). In luteal-phase ewes, central kisspeptin infusion increased LH pulse frequency (Li et al. 2015). The effects of kisspeptin on LH pulsatility remain to be determined when administered during the pre-ovulatory or luteal phase of the menstrual cycle, when LH sensitivity to kisspeptin is heightened (Dhillo et al. 2007, Jayasena et al. 2011, Chan et al. 2012).

The ability of kisspeptin to increase pulsatile LH secretion has also been demonstrated in human reproductive disorders of low LH pulsatility, including in hypothalamic amenorrhoea (Jayasena et al. 2014b), in hypogonadal men with type 2 diabetes (George et al. 2013), and in defects in neurokinin B and its receptor (Young et al. 2013). It remains unclear as to whether kisspeptin administration can further enhance pulsatile LH release in states of high LH pulsatility in health, such as in postmenopausal women, and in disease, such as in PCOS.

1.2.6.6 Chronic or continuous exposure to kisspeptin

It is well established that continuing administration of GnRH desensitises the hypothalamic-pituitary-gonadal axis after an initial stimulation (Belchetz et al. 1978), the phenomenon of which is applied clinically in the management of sex-steroid dependent disorders and in IVF. Downregulation of gonadotropin-releasing hormone receptors and desensitization of gonadotropes is the mechanism by which GnRH analogue suppresses gonadotropin output (McArdle et al. 1987, Mason et al. 1994). Given that pulsatile (i.e. non-continuous) kisspeptin secretion has been observed within the median eminence of the monkey (Keen et al. 2008), the potential of prolonged kisspeptin exposure to bring about desensitisation has been

investigated, but it appears to be less clearly demonstrable than that of continuous GnRH administration.

Continuous administration of kp-10 (intravenous 200 µg/hr [154 nmol/kg] or 400 µg/hr [307 nmol/kg] for 98 hours) to rhesus monkeys resulted in suppressed LH secretion, indicative of kisspeptin receptor desensitization (Ramaswamy et al. 2007). Similarly, the stimulatory effect of kp-54 on LH and testosterone secretion was lost after 2 days of continuous subcutaneous administration at 50 nmol/day in male rats (Thompson et al. 2006). The kisspeptin receptor has been shown to desensitise *in vitro* by uncoupling from its G-protein coupled complex and undergoing internalization (Pampillo et al. 2009). Consistent with this, repeated subcutaneous administration of KP-54 (6.4nmol/kg twice daily) for two weeks in women with hypothalamic amenorrhea resulted in an initial stimulation of LH and FSH secretion which was not maintained, indicating that chronic nonpulsatile exposure of kisspeptin causes tachyphylaxis in humans (Jayasena et al. 2009) (Fig. 1.3). In those women, LH response to GnRH administration after KP-54 injections was preserved and comparable to GnRH-stimulated LH secretion before kisspeptin treatment, indicating that desensitisation to kisspeptin was at the hypothalamus and upstream of the pituitary gland (Jayasena et al. 2009), which is consistent with animal data (Seminara et al. 2006). Whilst continuous kp-10 infusion reduced the frequency and amplitude of LH pulses in rhesus monkeys (Ramaswamy et al. 2007), this was not changed after kisspeptin treatment in women with hypothalamic amenorrhoea (Jayasena et al. 2009). This discrepancy may be explained by rapid recovery from kisspeptin exposure in the latter as assessment of LH pulsatility was 24 hours post final kisspeptin injection, and a protocol using more than 40-fold higher dose of kisspeptin and for longer period of time in the former. Jayasena and colleagues have suggested there might be a dose-dependent window within which kisspeptin would sustain basal LH secretion and restore LH pulsatility. Intravenous KP-54 infusion at 1 nmol/kg/hr for 10 hours in women with hypothalamic amenorrhea increased LH secretion to peak at 5 hours with a gradual decline thereafter (Jayasena et al. 2014b). Similarly, detectable LH pulses were diminished after 4 hours of 1 nmol/kg/hr of kisspeptin administration (Jayasena et al. 2014b). Lower doses of KP-54

(intravenous 0.01-0.3 nmol/kg/hr for 8 hours) showed no desensitisation, and there was overall increase in the mean peak number and secretory mass of LH pulses (Jayasena et al. 2014b).

However other studies in healthy human subjects using infusions or repeated administration of kisspeptin have not provided consistent evidence for desensitization (Fig. 1.2). Kisspeptin-54 (6.4 nmol/kg) administered twice daily for a week advanced the menstrual cycle in healthy women (Jayasena et al. 2013a). The study did not continue KP-54 injections for 2 weeks to determine whether desensitisation would be observed as in women with hypothalamic amenorrhoea (Jayasena et al. 2009). It is possible that women with hypothalamic amenorrhoea are more sensitive to desensitisation by kisspeptin given that their immediate LH response to kisspeptin itself is greater than in follicular phase women, despite the low estrogenic environment. In healthy men, continuous KP-10 infusion at 4 µg/kg/hr (3.1 nmol/kg/hr) for 22.5 hours showed continuing stimulation of LH secretion, with no evidence of desensitisation (George et al. 2011) (Fig. 1.2). In contrast, LH secretion was not sustained in three healthy men during KP-10 infusion for 24 hours at 12 µg/kg/hr (9.2 nmol/kg/hr), the highest dose and the longest duration of infusion used in humans to date (Lippincott et al. 2013). However LH secretion remained well above baseline values at the end of infusion in contrast to marked desensitisation with KP-54 in women with hypothalamic amenorrhoea (Jayasena et al. 2009). It is yet to be determined if an infusion of kisspeptin for longer than 24 hours would unlimitedly result in LH suppression to castrate levels or maintain it above baseline.

Whilst continuous kisspeptin administration at high doses has the potential to induce desensitisation, intermittent administration sustains GnRH and LH pulsatility. In contrast to KP-54 administered twice daily for 2 weeks, the same dose of KP-54 (6.4 nmol/kg) injected twice weekly sustained the secretion of gonadotropins for 8 weeks after a brief initial suppression (Jayasena et al. 2010) (Fig. 1.3). Intermittent administration of kisspeptin-10 in juvenile male monkeys (intravenously hourly for 2 days) and juvenile female rats (intracerebroventricular twice daily for 5 days) caused precocious puberty, indicating that GnRH and LH were released in a pulsatile

manner, which is necessary for the physiological role of GnRH (Navarro et al. 2004b, Plant et al. 2006). In a dose-finding study of bolus KP-10 injection in healthy men, the highest dose (3 μg/kg [2.3 nmol/kg]) elicited a sub-maximal response compared to lower doses, suggesting that kisspeptin-10 can cause desensitisation even with bolus administration (George et al. 2011). An alternative explanation for this observation is that kisspeptin at the higher dose kisspeptin-10 might have stimulated another RF-amine receptor, such as gonadotropin inhibitory hormone receptor, known to have inhibitory effect on GnRH and LH (George et al. 2011).

These data suggest that while high doses of kisspeptin may induce desensitisation, evidence for tachyphylaxis in humans is inconsistent. There appears to be a fine balance in the ability of kisspeptin to upregulate or downregulate the hypothalamic-pituitary-gonadal axis. The discrepancies between the studies might reflect the duration of kisspeptin administration (8-22.5 hours vs 2-8 weeks), lower doses of kisspeptin infused in the human studies compared those in primate, variation in the isoform of kisspeptin used, the mode of kisspeptin administration, differences between the human and animal models and even health status (healthy volunteers versus women with hypothalamic amenorrhea).

Whilst tachyphylaxis caused by natural forms of kisspeptin is controversial, kisspeptin receptor agonist analogues, TAK-448 and TAK-683 are potent induces of desensitisation. Phase I clinical studies in healthy men showed that subcutaneous infusion of TAK-683 (0.01-2mg/day) (Scott et al. 2013) and TAK-448 (0.01-1mg/day) (MacLean et al. 2014) for 2 weeks suppressed testosterone below castration levels, the latter of which also reduced testosterone and prostate specific antigen in patients with prostate cancer (MacLean et al. 2014). Kisspeptin analogues may be advantageous compared to the GnRH analogues widely used today in that the effect of kisspeptin analogues was more rapid and profound in suppressing the hypothalamic-pituitary-gonadal axis, thereby limiting the initial stimulatory phase of gonadotropin secretion prior to downregulation that is characteristic of therapies using GnRH agonists (Matsui and Asami. 2014).

1.2.6.7 Sexual dimorphism in response to kisspeptin

Men and women display sexual dimorphism in their response to exogenous kisspeptin. Whilst kisspeptin potently stimulates the release of LH in men, the effect of kisspeptin is more variable in women and depends on the phase of the menstrual cycle (Fig. 1.2). The stimulatory potency of kisspeptin is greatest in the pre-ovulatory phase and least in healthy women in the early follicular phase of the menstrual cycle (Dhillo et al. 2007, Jayasena et al. 2011, Chan et al. 2012), which is consistent with data in rats (Roa et al. 2006) and sheep (Smith et al. 2009b). This variability in women may therefore reflect changes in the activity of the hypothalamic kisspeptin system and in the pituitary sensitivity to GnRH, with the varying sex steroid environment playing a major role in regulating this, which is discussed in more detail in section 1.2.6.1 (Stimulatory effect of kisspeptin on gonadotropin secretion).

The sexual dimorphism in the responsiveness of men and women has been elegantly illustrated using different isoforms of kisspeptin (Jayasena et al. 2011) (Fig. 1.2). Men respond to modest doses of both KP-54 and KP-10 and with broadly similar potencies of action on gonadotropin secretion when compared in equimolar doses (Dhillo et al. 2005, George et al. 2011, Jayasena et al. 2011, Jayasena et al. 2015a). In women, even the highest doses of KP-10 administered as an intravenous bolus (10 nmol/kg), subcutaneous bolus (32 nmol/kg) or an intravenous infusion (720 pmol/kg/min for 90 mins) failed to mount an LH response in the follicular phase (Jayasena et al. 2011). However in subsequent studies low-dose intravenous KP-10 bolus (0.24 nmol/kg) stimulated LH secretion in the early follicular phase (Chan et al. 2012, George et al. 2012). The discordance in results may have been methodological- the former study had no baseline LH sampling as opposed to the latter two studies, which employed a 10-minute LH blood sampling for 3 (George et al. 2012) and 6 hours (Chan et al. 2012) prior to kisspeptin administration, allowing comparison of LH secretion before and after KP-10 administration within the same subject. Differential FSH response to kp-10 has been suggested in rodents, with the response being more prolonged in male rats (Pinilla et al. 2012). KP-54 is more consistent in inducing LH secretion when administered as an intravenous or

subcutaneous bolus or a subcutaneous infusion in the follicular phase (Dhillo et al. 2007, Jayasena et al. 2011, Jayasena et al. 2013a, Jayasena et al. 2013b, Narayanaswamy et al. 2016a), suggesting that the response to the longer isoform of kisspeptin is substantially more robust, perhaps reflecting its longer half-life. This is consistent with studies in rodents where subcutaneous kp-54 stimulated LH secretion more potently in male rats compared to kp-10 (Thompson et al. 2006). Interestingly, it has been consistently found that in the pre-ovulatory phase both KP-54 and KP-10 potently stimulate gonadotropin secretion (Dhillo et al. 2007, Jayasena et al. 2011, Chan et al. 2012).

Sexual dimorphism has also been apparent in other aspects of kisspeptin signalling: there are marked differences in kisspeptin neurone abundance between males and females (Clarkson and Herbison. 2006, Kauffman et al. 2007, Cheng et al. 2010, Hrabovszky et al. 2010) (see section 1.2.5.4 Sexual dimorphism of kisspeptin neurones); spontaneous electrical firing of kisspeptin neurones is different in female to male mice (de Croft et al. 2012); female mice require higher frequency of stimulation to elicit action potential and LH secretion then males (Han et al. 2015); kisspeptin resets the hypothalamic GnRH clock in men but not in women (Chan et al. 2011, Jayasena et al. 2011) (see section 1.2.6.4 Kisspeptin as GnRH pulse generator). This sexual variation in the anatomical distribution of kisspeptin pathway and many aspects of kisspeptin-kisspeptin receptor physiology may reflect sexually dimorphic roles of kisspeptin, notably in the generation of the pre-ovulatory LH surge, which is unique to the female.

1.2.7 Kisspeptin and puberty

The obligate role of kisspeptin-kisspeptin receptor pathway in reproduction was established by genetic studies in human puberty undertaken simultaneously by two independent researchers, where 'loss of function' mutations in *KISS1R* were identified in patients with hypogonadotropic pubertal delay (de Roux et al. 2003, Seminara et al. 2003) (see section 1.2.3 Discovery of the role of kisspeptin in reproduction). Since these early reports, other 'loss of function' mutations in *KISS1R* have been reported (Tenenbaum-Rakover et al. 2007). A similar phenotype was later demonstrated in a large consanguineous family with all affected members bearing a homozygous mutation in *KISS1* gene itself (Topaloglu et al. 2012).

The clinical findings of kisspeptin as a crucial regulator of puberty have been supported by numerous studies in animals. *Kiss1r* and *Kiss1* deficient mice display a phenotype of human normosmic idiopathic hypogonadotropic hypogonadism, although they show variable reproductive potential ranging from complete reproductive phenotype knock-out (Seminara et al. 2003, d'Anglemont de Tassigny et al. 2007) to partial sexual maturation, indicating residual activity of the HPG axis (Chan et al. 2009a). In general, the phenotype of the *Kiss1r* knock-out mice is more severe compared to the *Kiss1* knock-out mice (Lapatto et al. 2007). Nevertheless, the reproductive abnormality appears to stem from the *Kiss1-Kiss1r* signalling as gonadotropin secretion is observed in response to kisspeptin and GnRH administration, respectively (Seminara et al. 2003, d'Anglemont de Tassigny et al. 2007). A GnRH neurone specific *Kiss1r* knock-out mice displayed pubertal delay with low gonadotropin levels and external abnormalities, including microphallus and decreased ano-genital distance in males, suggesting that disruption of kisspeptin receptor signalling alters gonadal steroid synthesis and secretion (Novaira et al. 2014). Likewise, central administration of kisspeptin antagonist delayed vaginal opening (a marker of pubertal induction in rodents) and decreased uterine and ovarian weights in peripubertal female rats (Pineda et al. 2010) and inhibited pulsatile GnRH secretion in pubertal female monkeys (Roseweir et al. 2009). Conversely, ablation of *Kiss1*-expressing cells did not affect pubertal onset in female mice(Mayer and Boehm. 2011). This contrasts with impaired reproductive phenotype

reported in *Kiss1* and *Kiss1r* deficient mice (Seminara et al. 2003, d'Anglemont de Tassigny et al. 2007, Lapatto et al. 2007, Chan et al. 2009a) and with complete absence of reproductive function in patients with inactivating *KISS1* and *KISS1R* mutations (de Roux et al. 2003, Seminara et al. 2003, Topaloglu et al. 2012) and may indicate incomplete kisspeptin neuronal ablation.

Mutations in kisspeptin signalling not only cause pubertal delay, but can also be associated with advancing pubertal onset. An 'activating' autosomal dominant point mutation in the *KISS1R* gene in an adopted girl with precocious puberty has been reported, although the inheritance could not be determined as the biological family was not available for genetic testing (Teles et al. 2008). To investigate this further, cells transfected with the mutant kisspeptin receptor showed slower desensitization and prolonged accumulation of inositol phosphate and phosphorylation of extracellular signal-regulated kinase, indicating extended intracellular signalling by the mutant kisspeptin receptor (Teles et al. 2008). Missense mutations have been subsequently described in the *KISS1* gene in association with central precocious puberty (Silveira et al. 2010). Functional characterisation studies showed this mutant kisspeptin to be more resistant to *in vitro* degradation, suggesting that greater kisspeptin bioavailability might be accounting for the precocious puberty (Silveira et al. 2010). A cohort of Korean girls with precocious puberty appeared to have less frequent polymorphism in *KISS1* gene that authors suggested might be otherwise protective against pubertal precocity (Ko et al. 2010) and had higher serum kisspeptin levels compared to healthy controls (Rhie et al. 2011). Interestingly, 6 months of pubertal suppression treatment with GnRH agonist resulted in lower serum kisspeptin levels in girls with precocious puberty when compared to pre-treatment levels (Demirbilek et al. 2012).

In addition to human studies, anatomical and functional experiments in animals, including primates, implicate the role of kisspeptin in the onset of pubertal maturation. Hypothalamic expression of *Kiss1* and *Kiss1r* mRNA is dramatically upregulated at puberty in rodents and primates (Navarro et al. 2004a, Han et al. 2005, Shahab et al. 2005, Clarkson and Herbison. 2006), and the percentage of GnRH neurones depolarising in response to kisspeptin increases from juvenile (25%) to

prepubertal (50%) to adult mice (over 90%), suggesting that GnRH neurones gradually acquire sensitivity to kisspeptin across puberty (Han et al. 2005). Kp-54 secretion increased in association with the pubertal increase in GnRH, and furthermore kp-54 release was pulsatile at approximately 60-minute intervals at the onset of puberty in female monkeys (Keen et al. 2008). Kp-10 infusion hourly for 48 hours in prepubertal male monkeys stimulated LH pulses that mimicked those induced by pulsatile synthetic GnRH administration (Plant et al. 2006) and were similar to LH pulses observed in castrated adult male monkey (Plant. 1982), suggesting GnRH-dependent kisspeptin signalling at puberty. Complementary to physiological changes linking kisspeptin and the onset of puberty, intracerebroventricular delivery of kisspeptin from postnatal day 26 to 31 induced precocious puberty in female rats, displaying advanced vaginal opening, increased uterine weighs and raised LH and estradiol secretion (Navarro et al. 2004b).

Taken together these data support the hypothesis that during juvenile period reduced kisspeptin stimulatory tone initiates the prepubertal brake, resulting in GnRH quiesecence, and it is the upregulation of the arcuate kisspeptin expression and increased kisspeptin release in the peripubertal state that reactivates pulsatile GnRH secretion (Herbison. 2016a). In females, the later development of pre-optic kisspeptin neurone inputs to GnRH in the pubertal period allows the generation of the GnRH surge (Herbison. 2016a). Kisspeptin-kisspeptin receptor interaction appears to be a necessity to initiate and progress through the puberty, although the exact mechanism by which kisspeptin orchestrates and likely interacts with other neuroendocrine modulators during the awakening of the HPG axis remains to be elucidated.

1.2.8 Kisspeptin and metabolism

Reproductive function is influenced by metabolic status with both extremes of nutrition –starvation and obesity having a negative impact on reproduction across the species. Kisspeptin has been implicated as an intermediary link between energy homeostasis and reproduction by sensing energy stores and translating this information into the pulsatile GnRH secretion. Food-deprived rodents and non-human primates show reduced expression of hypothalamic *Kiss1* mRNA and low gonadotropin levels with pubertal arrest in the former (Castellano et al. 2005, Cota et al. 2006, Roa et al. 2009, Wahab et al. 2011). Kisspeptin administration was able to restore delayed vaginal opening and normalised low gonadotropin and estrogen levels associated with undernutrition in pre-pubertal rats (Navarro et al. 2004b, Castellano et al. 2005). However, testosterone response to peripheral kisspeptin injection was delayed and suppressed in mature male monkeys following 18- and 24-hour fasting-induced metabolic deficiency (Wahab et al. 2008, Wahab et al. 2014), suggesting that fasting-induced suppression of the reproductive axis may involve decreased responsiveness to endogenous kisspeptin.

Kisspeptin appears to mediate its role in energy homeostasis through interactions with leptin, the 'satiety hormone' of adipose tissue. Leptin has been show to activate GnRH neurones (Quennell et al. 2009), and deficiency of leptin is associated with pubertal delay and hypogonadotropic hypogonadism in mice and humans (Chehab et al. 1996, Clement et al. 1998). Whist GnRH neurones lack the leptin receptor (Ob-Rb), the arcuate kisspeptin neurones express this receptor, suggesting the role of kisspeptin in mediating the metabolic signals of leptin on the HPG axis (Smith et al. 2006a). In animals subject to negative energy balance, low serum leptin levels results in decreased expression of *Kiss1* mRNA (Castellano et al. 2005, Smith et al. 2006a, Wahab et al. 2011), which is subsequently upregulated by leptin (Smith et al. 2006a, Backholer et al. 2010). In sheep, this has been observed in both kisspeptin populations at the pre-optic region and the arcuate nucleus (Backholer et al. 2010). Interestingly, in leptin-deficient mice *Kiss1* mRNA expression is not completely restored in response to leptin (Smith et al. 2006a). Partial recovery of kisspeptin signalling in response to leptin is consistent with 40% of kisspeptin neurones

localising to the leptin receptors and indicate the involvement of other mediators in suppressing kisspeptin pathway in leptin deficiency (Smith et al. 2006a). Indeed, mice with selective deletion of leptin receptor from kisspeptin neurones undergo normal pubertal maturation, sexual development and are fertile, suggesting that kisspeptin pathway is not critical for leptin signalling (Donato et al. 2011).

Kisspeptin appears to replay the metabolic cues of other hypothalamic peptides. The arcuate kisspeptin neurones are in close association with neuropeptide Y (NPY) and pro-opiomelanocortin (POMC) neurones (Backholer et al. 2010). NPY is an orexigenic peptide, which decreases pulsatile LH secretion (Barker-Gibb et al. 1995), whilst POMC (a precursor for α-melanocyte-stimulating hormone (α-MSH)) is anorexigenic and stimulates the activity of kisspeptin and increases LH secretion (Backholer et al. 2009). Leptin receptor is also expressed by NPY and POMC neurones and may indirectly inform kisspeptin neurones on the body energy levels (Iqbal et al. 2001).

Kisspeptin is also involved in mediating metabolic cues of gut hormones, in addition to adipose hormones, all of which interact with one another. For example ghrelin, the gut 'hunger hormone', is elevated under food-deprived conditions and is shown to decrease *Kiss1* transcripts (Forbes et al. 2009). The role of gut and adipose hormones as regulators of energy homeostasis and their interaction with the reproductive axis is discussed in detail elsewhere (Comninos et al. 2014).

Secondary hypogonadism has been observed in men with obesity and type 2 diabetes, where decreased pulsatile GnRH secretion is thought to be the common factor (Dandona et al. 2008). However, the central cause of reproductive impairment appears to be upstream of GnRH as gonadotropin response exogenous kisspeptin administration is preserved in human diabetic patients and experimental diabetic animals (Castellano et al. 2006b, Castellano et al. 2009) (George 2013). A rat model of diabetes (streptazocin treated) display reduced levels of *Kiss1* mRNA with subsequently low levels of circulating gonadotropins and sex steroids, which are corrected by kisspeptin (Castellano et al. 2006b, Castellano et al. 2009). Similarly, obese rats had decreased hypothalamic *Kiss1* mRNA expression, low LH and testosterone levels, and suppressed kisspeptin-induced LH secretion (Sanchez-

Garrido et al. 2014). This suggests that lack of stimulatory kisspeptin drive to the hypothalamic GnRH cells is a potential mechanism for the downregulation of reproductive axis seen with obesity and diabetes (George et al. 2010). Indeed, intravenous infusion of KP-10 (4 μg/kg/hr [3.1 nmol/kg/hr] for 11 hours) in hypogonadal men with type 2 diabetes increased the frequency and mass of LH pulses and LH and testosterone secretion (George et al. 2013) (Fig. 1.3). Low levels of leptin as opposed to insulin deficiency/resistance appears to be responsible for suppressed kisspeptin signalling in diabetes, as an administration of leptin and not insulin enhanced kisspeptin expression in the mouse hypothalamic cell line (Luque et al. 2007). Insulin receptors are expressed in the arcuate kisspeptin neurones, suggesting some role of insulin-kisspeptin interaction. Indeed, mice lacking insulin receptor from kisspeptin neurones showed signs of delayed puberty but otherwise normal adult reproductive capacity (Qiu et al. 2013). George and colleagues (George et al. 2010) have suggested that the mechanisms leading to suppression of kisspeptin signalling and subsequent hypogonadotropic hypogonadism in diabetes are multi-factorial and include: upregulation of negative estrogen feedback (Schneider et al. 1979); resistance to leptin seen in human obesity (Finn et al. 1998); insulin resistance and hyperglycaemia (Castellano et al. 2006b, Castellano et al. 2009); and inflammation, which is upregulated in hypogonadal men with diabetes (Dandona et al. 2008) and is associated with decreased kisspeptin expression in rats (Iwasa et al. 2008).

1.3 Neurokinin B- the upstream gatekeeper of kisspeptin-GnRH pathway

The hypothalamic peptide neurokinin B (NKB) is now established as an essential regulator of pubertal acquisition and normal adult reproductive function across species, including humans. Neurokinin B is believed to act predominantly upstream of kisspeptin and signals via the neurokinin-3 receptor (NK3R) to mediate its stimulatory (although with some discordance in rodent data) effects on pulsatile GnRH and thus gonadotropin secretion. Co-expression of neurokinin B by the same functional neuronal network that also co-expresses kisspeptin and dynorphin (KNDy neurones) in the arcuate nucleus and their intimate interconnection to each other, indicates a complex interaction between the three neuropeptides and a degree of self-regulation via the NK3R and kappa opioid receptor but not the kisspeptin receptor expressed within KNDy cells. Close apposition of KNDy projections to GnRH nerve terminal at the median eminence raises the possibility of this network being at the core of the GnRH pulse generator with NKB-NK3R signalling as an important component.

1.3.1 Nomenclature and signalling

Neurokinin B belongs to the tachykinin family peptides (10-11 amino acids), which share a common C-terminal amino acid sequence (Phe-X-Gly-Leu-Met-NH_2) and include substance P, neurokinin A, neuropeptide K, neuropeptide γ, and hemokinin-1 (Page. 2005). Neurokinin B is encoded by the *TAC3* gene in humans, equivalent to *Tac3* in non-human primates, cattle and dogs, and *Tac 2* in rodents. Five out of seven exons of the *TAC3* gene are first translated into the precursor preprotachykinin-B, which is then cleaved to proneurokinin B and finally to neurokinin B (Bonner et al. 1987, Page et al. 2009). *NKB/TAC3/Tac2* mRNA will be used when referring to the mRNA transcripts of this gene.

The cognate receptor for neurokinin B is NK3R, encoded in five exon *TACR3* gene in humans and *Tacr3* in rodents (Almeida et al. 2004). NK3R belongs to the rhodopsin-like family of G-protein coupled receptor with seven hydrophilic transmembrane domains (Almeida et al. 2004). Tachykinin family also has other

receptors, NK1R and NK2R for which substance P and neurokinin A are the preferential ligands, respectively, although each, including neurokinin B, can activate other three receptors (NK1R, NK2R and NK3R) (Regoli et al. 1994, Pennefather et al. 2004). Upon binding of neurokinin B to its NK3R, it can activate two intracellular signalling pathways- via phospholipase C, which recruits inositol triphosphate to increase calcium influx into the cells, and/or via adenylate cyclase to yield cAMP concentrations (Satake and Kawada. 2006).

1.3.2 Neuroanatomy of NKB/NK3R pathway

1.3.2.1 Distribution of NKB within the brain

The distribution of *NKB* mRNA expressing neurones has been mapped in detail in the human hypothalamus and basal forebrain (Chawla et al. 1997). Neurokinin B neurones are predominately located in the infundibular nucleus (Chawla et al. 1997), which is conserved across species and localised to the arcuate nucleus (equivalent to the human infundibular nucleus) in rat (Marksteiner et al. 1992), mouse (Duarte et al. 2006), sheep (Foradori et al. 2006), and monkey (Sandoval-Guzman et al. 2004). Small numbers of neurokinin B neurones are also scattered in other regions of the brain, including septal region, band of Broca, bed nucleus of the stria terminalis and amygdala in humans (Chawla et al. 1997) and rats (Marksteiner et al. 1992), although species difference exist, as for example expression of *Tac2* mRNA in the rat hippocampus and olfactory bulb is absent in mice (Duarte et al. 2006).

The key relevant feature of the infundibular/arcuate neurokinin B neurones is their co-localisation with kisspeptin and dynorphin in numerous species, including humans (Goodman et al. 2007) (see section 1.2.5.2 Kisspeptin neurones co-express other neuropeptides). Studies using dual immunocytochemistry and track-tracing have identified dense neuronal network of NKB/dynorphin in the rat arcuate nucleus, the axons of which are in close apposition to NKB/dynorphin cell bodies and dendrites, suggesting close communication between those neurones (Burke et al. 2006). The arcuate neurokinin B neurones branch out to and are in close apposition to the GnRH terminals at the median eminence (Goubillon et al. 2000, Krajewski et al. 2005, Ciofi et al. 2006), and extend across it to the contralateral arcuate nucleus in

rats (Krajewski et al. 2010) as well as other regions of the brain, including the AVPV nucleus and the medial pre-optic area, the dorsomedial hypothalamus and the lateral hypothalamic areas (Burke et al. 2006, Krajewski et al. 2010, Rance et al. 2010).

1.3.2.2 Distribution of NK3R within the brain

NK3R is expressed in the arcuate nucleus and furthermore is present on NKB/kisspeptin/dynorphin cells (Krajewski et al. 2005, Navarro et al. 2009), suggesting that neurokinin B may exert feedback on reciprocally interconnected neurokinin B-expressing neurones in the regulation of kisspeptin and subsequently GnRH secretion. Generally, regions receiving the arcuate neurokinin B fibres in rat also express NK3R (Krajewski et al. 2005). NK3R-immunoteactivity was detected on GnRH terminals in the median eminence in rodents (Krajewski et al. 2005, Todman et al. 2005), suggesting direct action of neurokinin B on GnRH release. However, neurokinin B cells in rats did not take up intraperitoneally administrated aminostilbamidine, a retrograde tracer that does not cross the blood-brain-barrier, questioning their localisation and therefore function at the fenestrated capillaries at the median eminence having access to the blood-borne particles (Krajewski et al. 2005). NK3R localisation to GnRH neurones has not been investigated in other species.

1.3.2.3 Sexual dimorphism of neurokinin B neurones

Similarly to the kisspeptin neuronal network, gender differences are present in the numbers and morphology of neurokinin B neurones. Ewes have increased numbers of neurokinin B neurones in the arcuate nucleus compared to rams and females androgenized prenatally, suggesting that this sex difference is influenced by testosterone early in development (Goubillon et al. 2000, Cheng et al. 2010). There is also sexual dimorphism in the distribution of neurokinin B axons in the rat arcuate nucleus, where in males, neurokinin B axons are in close proximity to the fenestrated capillaries in the median eminence, whereas in females, neurokinin B axons are more diffusely distributed (Ciofi et al. 2006). Postnatal development of the hypothalamic neurokinin B neuronal network is distinct in males and females in rats (Ciofi et al.

2007). Whilst in females, there appears to be progressive development in neurokinin B system (organised gathering of axons and increase in their numbers) from postnatal period (day 10) until puberty, they did not develop in males until puberty (day 40) (Ciofi et al. 2007). Furthermore, orchidectomy in prepubertal rats prevented and treatment with testosterone rescued masculine distribution of neurokinin B axons, whilst after estradiol administration the distribution of neurokinin B network was phenotypically female (Ciofi et al. 2007). In prepubertal ovariectomised female rats, testosterone exposure induced male-pattern of neurokinin B axon wiring (Ciofi et al. 2007). This shows that maturation of neurokinin B pathway is gender specific and furthermore is dependent on gonadal steroid exposure well beyond the perinatal period. Finally, the response of neurokinin B neurones to castration is different in prepubertal female and male rats. Ovariectomy was associated with an immediate increase in LH secretion and upregulation of the arcuate neurokinin B transcripts, whilst no changes were observed in LH levels and the expression neurokinin B in castrated juvenile males (Kauffman et al. 2009). This implies a gonadal-dependent suppression of the HPG axis before puberty in female rats but a different mechanism maintaining the quiescence of the gonadotropic axis in male rats (Kauffman et al. 2009).

1.3.3 Discovery of the role of neurokinin B in reproduction

Mechanistic studies: changes in NKB neurones in menopause in humans and animal models

The role of neurokinin B in the reproductive axis was first suggested by neuroanatomical studies comparing the expression of neurokinin B in the hypothalamus of pre- and postmenopausal women (Rance and Young. 1991). In postmenopausal women, the subset of neurones in the infundibular nucleus was hypertrophied and expressed *NKB* mRNA and *ERα* mRNA (Rance et al. 1990, Rance and Young. 1991). Furthermore, there was marked increase in *NKB* mRNA expression of those hypertrophied neurones when compared to premenopausal women (Rance et al. 1990, Rance and Young. 1991). This suggests a role of neurokinin B in mediating negative estrogen feedback, whereby loss of steroid negative feedback with ovarian follicle exhaustion at the menopause results in heightened neurokinin B activity and high gonadotropin secretion.

The involvement of neurokinin B in the control of the HPG axis was subsequently supported by animal findings, where ovariectomy increased and estrogen replacement reduced neurokinin B gene expression in monkeys (Abel et al. 1999, Sandoval-Guzman et al. 2004). In aging monkeys with low estrogen, resembling the human menopause, neurokinin B was unregulated in the arcuate nucleus (Eghlidi et al. 2010). Similarly, long-term (approximately 4 year) ovariectomy increased neurokinin B expression, which was reversed by estradiol treatment, whilst the expression of NK3R was not changed (Eghlidi et al. 2010).

Clinical studies: hypogonadotropic hypogonadism due to NKB/NK3R mutations

It was in 2009, when Topaloglu reported that loss of function mutations in the genes encoding neurokinin B (*TAC3*) and NK3R (*TACR3*) was associated with hypogonadotropic failure of pubertal progression in humans that firmly established the critical role of neurokinin B in normal function of the HPG axis (Topaloglu et al. 2009). In cell culture, NK3R mutants had diminished ability to mediate intracellular calcium increase in response to neurokinin B (Topaloglu et al. 2009). Male patients with these mutations show microphallus, suggesting inadequate neurokinin B action

and testosterone secretion in utero (Topaloglu et al. 2009). Since then a number of cases of similar phenotype have been reported with NKB/NK3R mutations, both homozygous and hererozygous, with a suggested prevalence of 5.5% amongst patients with normosmic hypogonadotropic hypogonadism (Gianetti et al. 2010, Rance et al. 2010).

In contrast to profound hypogonadotropic hypogonadism seen in patients with mutations in the neurokinin B pathway, rodents with genetic ablation of *Tacr3* do not show GnRH deficient phenotype and are fertile (Kung et al. 2004). *Tac2* (homologous to human *TAC3*) knock-out mice have not yet been described. However, the expression of Tac2/Tacr3 was upregulated in the rat hypothalamus prior to pubertal maturation (Gill et al. 2012, Navarro et al. 2012), and intraperitoneal injection of neurokinin B agonist advanced pubertal onset as manifested with earlier vaginal opening and first estrus in female rats (Nakahara et al. 2013). Moreover, chronic central infusion of NK3R antagonist delayed the time of vaginal opening in pubertal rats, although this was not as effective as with kisspeptin antagonism (Pineda et al. 2010). This suggest that whilst neurokinin B is involved, it may not be critical for the pubertal maturation in rodents, further highlighting species difference in the neurokinin B and moreover, KNDy pathway.

It is postulated that inability of neurokinin B to stimulate kisspeptin secretion in an autocrine and /or paracrine manner results in low GnRH pulse frequency with correspondingly low LH and gonadal steroid levels, but normal or near-normal levels of FSH typically seen in these patients. This pattern of gonadotropin secretion is reproduced by slow frequency of GnRH administration in rhesus monkeys (Wildt et al. 1981). Pulsatile administration of GnRH to adults with *TAC3* and *TACR3* mutations normalised serum LH and sex steroids and in one female resulted in live birth (Young et al. 2010). This indicates that infrequent GnRH pulse pattern is at the core of defective NKB/NK3R signalling.

Abnormal gonadotropin secretion in patients with mutations in the neurokinin B system appears to result from deficiency in the stimulatory action of kisspeptin on GnRH neurones. Indeed, continuous infusion of KP-10 (1.1nmol/kg/hr for 12 hours) in two patients with *TAC3* and two patients with *TACR3* mutations increased LH

response 2.5-fold and LH pulse frequency from none or very low (0.1 pulse/hour) to 0.5 pulses/hour (Young et al. 2013) (Fig. 1.3), close to the normal pattern of 0.7 pulses/hour seen in healthy men (George et al. 2011). This suggests that neurokinin B is essential for normal pulsatile GnRH secretion, and that the action of neurokinin B is upstream of kisspeptin. Overall, the LH response to kisspeptin was more limited and of lower LH mass per pulse than that observed in healthy men using the same kisspeptin administration regimen (George et al. 2011), consistent with a complex interaction of KNDy peptides rather than a linear hierarchy (Skorupskaite et al. 2014, Skorupskaite et al. 2016). Nevertheless, the significant increase in testosterone levels in male patients and estradiol levels in single female patient reflects an increase in LH and FSH following kisspeptin stimulation, although gonadal steroid levels were not corrected to the physiological range (Young et al. 2013). The authors explained partial restoration of gonadotropin and sex-steroid secretion in those patients by suggesting that while kisspeptin induces GnRH pulsatility readily, there is a delay in gonadotrope function which might be corrected with longer kisspeptin infusion and GnRH priming (Young et al. 2013).

Interestingly, some patients with NKB/NK3R deficiency demonstrate reversibility of hypogonadotropic hypogonadism with pulsatile pattern of GnRH secretion in adult life after discontinuation of sex-steroid or gonadotropin replacement, including two spontaneous conceptions and a further woman with regular menstruation (Gianetti et al. 2010). It is possible that the phenotype is rescued by other tachykinins, such as substance P, which is co-expressed by ERα-positive neurokinin B neurones in the infundibular nucleus (Rance and Young. 1991). However, the reported rates of reversibility in patients with mutations in neurokinin B signalling of around 80% (Gianetti et al. 2010) and 15% (Topaloglu et al. 2009) are notably higher than the 10% rate observed in a large cohort of other forms of normosmic hypogonadotropic hypogonadims cases (Pitteloud et al. 2005, Raivio et al. 2007). It is evident that NKB/NK3R pathway is a necessity for normal activation of HPG axis prenatally and at puberty, but once sexual maturation is achieved, neurokinin B signalling may not be required for sustained activity of the GnRH pulse generator (Topaloglu and Semple. 2011).

1.3.4 Effect of neurokinin B on GnRH and gonadotropin secretion

1.3.4.1 Neurokinin B response on GnRH/ LH secretion and pulsatility

Despite the critical role of neurokinin B in the onset of human puberty, its involvement in other aspects of reproduction is less clear. Administration of neurokinin B or a potent and selective NK3R agonist, senktide, has resulted in both stimulatory and inhibitory effects on LH secretion, depending on the animal model and gonadal status (Billings et al. 2010, Ramaswamy et al. 2010, Navarro et al. 2011a). Initial studies in rodents suggested an inhibitory action of neurokinin B on LH secretion when injected centrally in ovariectomised rats (Sandoval-Guzman and Rance. 2004) and mice (Navarro et al. 2009). Decreased LH pulse frequency (a marker for GnRH pulsatility) by intracerebroventricular administration of senktide in rats suggests that neurokinin B alters pulsatile GnRH secretion (Grachev et al. 2012). In another study, neurokinin B had no effect on LH secretion when administered in intact mice or in hypothalamic explants from rats *ex-vivo* (Corander et al. 2010). Subsequently, NK3R agonist-induced LH secretion was demonstrated in female rats in both diestrus and proestrus (Navarro et al. 2011a). The effect of neurokinin B on LH secretion in rodents appears to depend on the level of circulating estradiol levels, as neurokinin B is inhibitory in the absence of sex-steroids (ovariectomised rats), but stimulatory in the presence of physiological levels of estradiol (intact or estrogen-replaced ovariectomised rats) (Navarro et al. 2011a).

The situation is however different in higher species, including non-human primates, where the stimulatory action of neurokinin B on LH secretion is more consistent. Central administration of neurokinin B and/or its receptor agonist dramatically increased LH secretion to levels close to those observed during the pre-ovulatory LH surge in ewes (Billings et al. 2010) and stimulated LH pulse frequency (Li et al. 2015). Increased LH secretion was shown in the follicular phase and in anestrous ewes but not during the luteal phase, suggesting that response to neurokinin B is sex-steroid dependent consistent with ERα-expressing NKB neurones (Billings et al. 2010). Similarly, neurokinin B stimulated LH secretion in ovariectomised goats only

after pre-treatment with both estrogen and progesterone (Wakabayashi et al. 2010). Pre-treatment with GnRH receptor antagonist attenuated neurokinin B-induced LH discharges in agonadal prepubertal male monkeys, demonstrating that neurokinin B mediates its gonadotropic effects at or above the GnRH neurone (Ramaswamy et al. 2010). Whilst neurokinin B elicits acute LH response, repetitive stimulation of neurokinin B pathway by an intermittent infusion of senktide (for 1 minute hourly for 4 hours), in contrast to kisspeptin, blunted LH secretion in agonadal juveline male monkeys, suggestive of tachyphylaxis (Ramaswamy et al. 2010). Kisspeptin stimulation of LH release was preserved following downregulation with repeated senktide administration, suggesting that desensitisation to neurokinin B is likely to occur upstream of kisspeptin/GnRH interactions (Ramaswamy et al. 2010, Ramaswamy et al. 2011). Taken together, these data suggest sex-steroid modulation of neurokinin B action on LH secretion, and that it is upstream of and most likely via GnRH secretion, whereas discrepancies with rodent data may imply important interspecies variation in the neuroanatomical functioning of neurokinin B signalling.

Neurokinin B was more recently administered to humans for the first time (Jayasena et al. 2014c). Perhaps surprisingly, intravenous infusion of neurokinin B (highest dose 5.12 nmol/kg/hr for 8 hours) had no effect on LH, FSH and sex-steroid levels, and did not alter the frequency and mass of LH pulses in healthy men and women even when examined across different phases of menstrual cycle (Jayasena et al. 2014c). The discrepancy between human and animal findings might be explained by different doses (30 nmol/kg in monkeys versus 5.2 nmol/kg/hr in human) and routes of neurokinin B administration (central versus peripheral), variable sex-steroid feedback effects (intact versus post gonadectomy), species-specific action of neurokinin B and the interaction between the neurokinin B and other peptides of KNDy neurones (see section 1.3.4.2 The Interactions between KNDy neuropeptides).

Studies using neurokinin B antagonism have further strengthened our understanding of the hypothalamic control of gonadotropin secretion by neurokinin B. Central infusion of an NK3R antagonist (MRK-08) had a powerful suppressive effect on LH secretion and abolished both the frequency and amplitude of LH pulses in ovariectomised ewes, suggesting that neurokinin B signalling is involved in the

regulation of pulsatile GnRH and therefore LH secretion (Li et al. 2015). Similarly, in castrate monkeys oral preparation of an NK3R antagonist (ESN364) supressed LH but not FSH secretion, indicative of preferential suppression of LH levels by reduced GnRH pulsatility (Fraser et al. 2015a). When administered in sexually mature female monkeys from menstrual cycle day 2 for 35 consecutive days, neurokinin B antagonism (using the drug ESN364) had no effect on basal gonadotropin secretion but delayed surge-like LH and FSH secretion and decreased ovarian hormone response, although not to castrate levels (Fraser et al. 2015a).

In a recent randomised, double blinded, placebo-controlled clinical trial administering NK3R antagonist (AZD4901) in women with PCOS, 7 day administration of the highest dose of NK3R antagonist (40 mg oral twice daily) decreased LH secretion and subsequently circulating testosterone levels (George et al. 2016). NK3R antagonist interfered with GnRH pulse frequency as LH pulsatility was also reduced (George et al. 2016). Longer duration of treatment is required to establish the therapeutic application of neurokinin B antagonism in the treatment of hirsutism and potential resumption of menstrual regularity in PCOS. However, the inhibitory effect of neurokinin B antagonism on pulsatile GnRH/LH secretion has only been assessed when LH is hypersecreted and in disease models.

During the preparation of this thesis, data on NK3R antagonism emerged in healthy men and women. NK3R antagonist (ESN364; 20, 60 and 180 mg on day 1) decreased LH but not FSH secretion in men and women and subsequently testosterone secretion in men, although this effect was very short-lived post dosing with both hormones recovering within 24 hours (Fraser et al. 2016). In women, there was no overall decrease in basal LH secretion during treatment started in the follicular phase for 21 days, but NK3R antagonist delayed the LH surge in some women, probably as a consequence of a delayed pre-ovulatory estradiol rise (Fraser et al. 2016). However, the mechanism by which neurokinin B antagonism suppresses sex hormones remains unclear and direct gonadal effects cannot be ruled out since *TAC3/TACR3* and *KISS1/KISS1R* systems are expressed in the human ovary (Cejudo Roman et al. 2012, Garcia-Ortega et al. 2014). Although the action of NK3R

antagonist is consistent with slowing down the frequency of GnRH/LH pusatility, there are no data on this in states of high and low LH output in health.

1.3.4.2 The interactions between KNDy neuropeptides

It is now well accepted that kisspeptin, neurokinin B, and dynorphin of the same functional unit (KNDy neurones) in the human infundibular and the arcuate nucleus of other species interact to regulate GnRH and subsequently gonadotropin secretion, evidence for which is substantiated by both anatomical and functional data. In the only human study to date investigating the functional hierarchy of kisspeptin and neurokinin B, infusion of KP-10 was able to restore pulsatile LH secretion in men and women with inactivating mutations in neurokinin B (*TAC3*) and its receptor (*TACR3*), indicating that neurokinin B is functionally upstream of kisspeptin in regulating GnRH/LH secretion in humans (Young et al. 2013). This is supported by studies using *Kissr1* knockout mice, which were unable to show a stimulatory effect of NK3R agonist senktide on LH secretion (Garcia-Galiano et al. 2012). Central administration of senktide in female rats was associated with a 10-fold upregulation in c-fos (a marker of neural activity) expression in the arcuate *Kiss1* neurones as well as an increase in serum LH, suggesting that neurokinin B signalling can activate kisspeptin cells (Navarro et al. 2011a). Pre-treatment with NK3R antagonist abolished LH response to senktide but did not abrogate KP-10 induced LH secretion in GnRH primed juvenile male monkeys (Ramaswamy et al. 2010). Although NK3R is not widely expressed on GnRH neurones, its receptors are expressed by KNDy cells, where neurokinin B acts to regulate GnRH secretion (Krajewski et al. 2005, Navarro et al. 2011b). Current understanding is that the stimulatory neurokinin B and the inhibitory dynorphin via the cognate NK3R and the kappa opioid receptors expressed by KNDy cells autosynaptically coordinate the release of downstream kisspeptin, which through the kisspeptin receptor in turn drives the pulsatile secretion of GnRH and LH (Skorupskaite et al. 2014).

To further investigate the KNDy hypothesis, a very recent study for the first time co-administered kisspeptin, neurokinin B and naltrexone (an opioid receptor antagonist) in healthy men (Narayanaswamy et al. 2016c). Although all those treatments are

proposed to be stimulatory, divergent effects on gonadotropin secretion and pulsatile LH secretion were observed when they were co-administered. Generally, all kisspeptin (KP-54 0.1 nmol/kg/hr iv for 8 hours) and naltrexone (oral 50 mg) treated groups elicited pulsatile LH secretion, but those treated with neurokinin B (2.56 nmol/kg/hr iv for 8 hours) had variable results (Narayanaswamy et al. 2016c) (Fig. 1.2). Co-administration of neurokinin B limited the stimulatory effect of KP-54 on LH, FSH and testosterone secretion compared to KP-54 alone (Narayanaswamy et al. 2016c). This phenomenon has been documented in limited animal studies, where co-administration of neurokinin B with kisspeptin abrogated kisspeptin-induced GnRH secretion in hypothalamic explants from rats (Corander et al. 2010), although when injected centrally the stimulatory LH response to both neuropeptides was greater compared to kisspeptin alone (Corander et al. 2010). Incomplete access to the central nervous system with peripheral administration of neuropeptides may account for lowered response in the presence of neurokinin B, although the arcuate nucleus is thought to have some permeability to the blood-brain-barrier and its KNDy neurones project to the median eminence, which is outside this barrier. However, the expression *GnRH1* and *Kiss1r* was downregulated by the intra-arcuate administration of senktide in rats (Grachev et al. 2012). There is a possibility that neurokinin B acts to inhibit kisspeptin-induced GnRH and subsequently gonadotropin secretion, in the situations where kisspeptin signalling is high (Narayanaswamy et al. 2016c).

It has been postulated that the inhibitory effects of neurokinin B might be mediated by dynorphin. Dynorphin antagonism prevented senktide-induced suppression in LH secretion in ovariectomised rats with and without estradiol replacement (Grachev et al. 2012, Kinsey-Jones et al. 2012). However, no effect on gonadotropin secretion was observed with co-administration of naltrexone and neurokinin B in men (Narayanaswamy et al. 2016c). The inhibitory opioid action has also been implicated in the control of kisspeptin induced LH secretion. Naltrexone and kisspeptin treatment in men was the only combination that significantly increased the amplitude of LH secretion (Narayanaswamy et al. 2016c).

Taken together is it clear that there is a complex interaction rather than a linear hierarchy between the neuropeptides associated with KNDy neurone function in the

control of pulsatile GnRH secretion with further species differences operating at both anatomical and physiological levels. The KNDy system is highly sensitive to gonadal steroid status, which further modifies the signalling effects of those novel hypothalamic neuropeptides; this is discussed in more detail in section 1.4.1 Gonadal steroids.

1.3.4.3 Neurokinin B and the GnRH pulse generator

There are several lines of evidence to imply that infundibular/arcuate neurokinin B is a component of the GnRH pulse generating mechanism. The arcuate neurokinin B/kisspeptin/dynorphin neurones are the potential source of the multiunit volleys (MUA) of electrical activity that are associated with LH pulses, which are abolished by lesions in the arcuate nucleus in monkeys (Knobil. 1981). The role of neurokinin B on GnRH pulse generating activity was determined using MUA recording aimed at the arcuate KNDy neurones in estrogen-treated ovariectomised goats (Wakabayashi et al. 2010). A bolus of central administration of neurokinin B immediately evoked multiple MUA, lasting as long as 50 minutes after which spontaneous MUA volleys were re-established (Wakabayashi et al. 2010). Peripherally administrated senktide also induced MUA volleys and an accompanying pulse-like increase in LH secretion in ovariectomised goats (Yamamura et al. 2015). However, the efficacy of three neurokinin receptor-selective agonists was different with as little as 10 nmol of NK3R agonist potently inducing MUA volleys, whereas 100-fold higher doses of NK1R and NK2R agonist were effective in only some animals with no associated LH pulse observed in the absence of MUA (Yamamura et al. 2015). This suggests that the action of neurokinin B via the NK3R is predominantly involved in GnRH pulse generation, and the contribution of NK1R and NK2R is limited, if any, in goats (Yamamura et al. 2015). This is supportive by human data where inactivating mutations in *TAC3* and *TACR3* are associated with hypogonadotrpic hypogonadism and loss of LH pulsatility (Young et al. 2010).

A model of GnRH pulse generation has been proposed, where neurokinin B from the arcuate KNDy neurones acts autosynaptically via the NK3R on the same neurones as well as the neighbouring and contralateral KNDy neurones to coordinate and

synchronise electrical activity. This would evoke kisspeptin discharges, released at the GnRH terminal in the median eminence, where KNDy neurones are known to project to, which induce GnRH secretion. At the same time inhibitory dynorphin would terminate neurokinin B and kisspeptin release, thereby creating a kisspeptin pulse, which would translate into a pulse of GnRH(Navarro. 2013, Okamura et al. 2013). The involvement of dynorphin in the regulation of electrical KNDy neurone activity has also been demonstrated. Injection of dynorphin significantly increased the intervolley interval of the MUA, whereas kappa opioid antagonist was associated with decreased intervolley interval for the duration of the 2-hour infusion in ovariectomised goats(Wakabayashi et al. 2010).

Changes in the frequency and amplitude of LH pulsatility by stimulating or blocking NKB/NK3R signalling further supports the role of neurokinin B in control of the pulsatile GnRH secretion (Fraser et al. 2015a, Li et al. 2015, George et al. 2016, Narayanaswamy et al. 2016c). Neurokinin B had no effect on the orderliness of LH pulses as measured by approximate entropy (*ApEn*) in healthy men, whilst kisspeptin resulted in more ordered and naltrexone in more disordered LH pulses (Narayanaswamy et al. 2016c). Nevertheless, it is possible that neurokinin B is involved in generating an initial impulse to GnRH secretion, and kisspeptin is the final pathway, which after integrating the signals of neurokinin B and dynorphin, determines the rhythmicity of LH pulses.

1.3.5 Neurokinin B and metabolism

Given the involvement of the arcuate kisspeptin in conveying metabolic cues to the hypothalamus (see section 1.2.8 Kisspeptin and metabolism), it is reasonable to suspect that NKB/NK3R of the KNDy cells is also influenced by energy status in the metabolic regulation. Navarro and colleagues have studied metabolic influences on the neurokinin B control of puberty in rats. Animals subjected to 48 hours of fasting showed reduced *Tacr3* and to a lesser degree *Tac2* expression in their arcuate nucleus (Navarro et al. 2012). The NKB/NK3R pathway also appears to be more sensitive under fasting conditions with augmented LH response to senktide seen in pubertal rats (Navarro et al. 2012). Furthermore, repeated senktide administration was sufficient to induce vaginal opening and LH secretion in about half of the female rats with pubertal arrest secondary to malnutrition (Navarro et al. 2012).

The situation is reverse in cases of high caloric intake, where high fat diet was associated with precocious puberty in female rats (Li et al. 2012b). This was thought to be a result of premature upregulation of neurokinin B signalling since the expression of *Tac2* transcripts was increased in the arcuate nucleus, which in turn increased the frequency of LH pulsatility (Li et al. 2012b).

The effects of positive and negative energy balance on NKB/NK3R signalling are pretty much identical to the findings of kisspeptin on gonadotropin secretion (see section 1.2.8 Kisspeptin and metabolism). The arcuate KNDy neurone therefore appears to be the center that integrates central and peripheral cues on the metabolic status and conveys it to control the reproductive function via pulsatile GnRH secretion.

1.3.6 Neurokinin 3 receptor antagonism by AZD4901

Manipulation of the NKB signalling by antagonism of NK3R allows further exploration of a role of NKB in the regulation of reproductive function. A specific NK3R antagonist AZD4901 has been used in studies described in this thesis. AZD4901 is a high-affinity antagonist of the human NK3 receptor (Malherbe et al. 2011). It completely blocked neurokinin B agonist senktide induced calcium influx in human NK3R expressing Chinese hamster ovary cells (Malherbe et al. 2011).

It was initially developed for schizophrenia in 2007–2010 (as AZD2624) but did not meet its developmental efficacy goals for that indication (Litman et al. 2014). This NK3R antagonist, however, reduced testosterone and to some degree LH concentrations in healthy male volunteers (Malherbe et al. 2011). Studies using AZD4901 were not pursued further, as at the time, a reproductive role for neurokinin B was yet to be elucidated (Topaloglu and Semple. 2011). Since then much evidence has accrued suggesting that NKB has a central role in regulating GnRH and thus LH pulsatility (Pinilla et al. 2012).

Pharmacokinetic properties have been investigated in healthy men (Malherbe et al. 2011). AZD4901 is quickly absorbed following oral dosing. The elimination half-life for the drug is approximately 8.5 hours (Malherbe et al. 2011, Litman et al. 2014). Both the area under the concentration-time curve and maximum observed concentration (C_{max}) appears to be proportional to dose of AZD4901 with peak concentrations achieved at 1-8 hours (Malherbe et al. 2011, Litman et al. 2014). Renal elimination of this NK3R antagonist was negligible. With multiple dose administration, steady state was achieved in 4 days, and there appears to be no difference in pharmacokinetic properties between females and males (Malherbe et al. 2011). To date, there has been no clear demonstration of C_{max}-related toxicity in humans receiving up to 80mg of AZD4901 and this is the highest dose yet administered to humans. Data obtained from healthy volunteers and in women with PCOS did not identify any safety or tolerability concerns (Malherbe et al. 2011, George et al. 2016).

1.4 Other regulators of GnRH and gonadotropin secretion

1.4.1 Gonadal steroids

The role of sex-steroids in the control of pulsatile GnRH and subsequently gonadotropin secretion in humans was proposed in early 1970s, when in healthy women across different phases of the menstrual cycle a variable pattern of gonadotropin release was observed with a pulse and a mid-cycle surge mode of LH/FSH secretion (Midgley and Jaffe. 1971). Subsequently, higher frequency of LH pulsatility was demonstrated in ovarian function deficient post-menopausal women compared to pre-menopausal women (Yen et al. 1972) with similar findings reproduced in ovariectomised sheep and monkeys (Dierschke et al. 1970, Bolt. 1971).

1.4.1.1 Estrogen, progesterone and testosterone feedback

Estrogen feedback

During the follicular phase of menstrual cycle, estrogen exerts negative feedback to limit GnRH activity and therefore LH secretion. However, in the late follicular phase, by yet unknown mechanisms negative estrogen feedback switches to positive estrogen action, culminating in the pre-ovulatory LH (and to a lesser extent FSH) surge needed for ovulation. Negative and positive estrogen control of gonadotropin secretion was initially demonstrated by variable duration of estrogen administration intramuscularly every 12 hours in the follicular phase women (Keye and Jaffe. 1975). Short-term estrogen treatment (36 hours) inhibited, whilst prolonged estrogen treatment (132 hours) augmented LH and FSH response to GnRH (Keye and Jaffe. 1975). Estrogen feedback modulates pulsatile GnRH and subsequently LH release. In post-menopausal women treated with intravaginal estrogen, LH pulse frequency decreased within 24 hours of estrogen treatment, then increased on day 5 and 10 of the study and declined thereafter (Veldhuis et al. 1987b). Similarly in men, estradiol infusion suppressed LH pulsatility and tamoxifen (a selective estrogen receptor modulator) had an opposite effect (Veldhuis and Dufau. 1987). Modulation of GnRH and gonadotropin secretion by estrogen is consistent with animal studies, where in rodents, intra-arcuate estrogen administration diminished LH pulse frequency

(Akema et al. 1984) and in monkeys, hypothalamic electrical activity was arrested with associated decreased in pulsatile LH secretion (Kesner et al. 1987), suggesting negative estrogen action on the hypothalamic GnRH pulse generator.

Portal blood vessel sampling studies in sheep during positive estrogen feedback showed a gradual increase in the frequency and amplitude of GnRH pulses, which ultimately culminates in a prolonged GnRH surge (Clarke. 1993). Estradiol has been shown to generate GnRH/LH surge in a number of mammals but only when administered at sufficiently high levels for a prolonged period of time, which in ewes has been suggested to be of a minimum 7-14 hours (Karsch and Foster. 1975, Moenter et al. 1990, Evans et al. 1997).

ERα is the predominant nuclear receptor involved in classical estrogen signalling via the estrogen response element (ERE) to alter gene transcription (Herbison. 2015). Mice deficient in ERα were unable generate positive feedback and a GnRH/LH surge (Glidewell-Kenney et al. 2007). On the other hand ERβ deficient mice were fertile, suggesting this isoform of estrogen receptor may not have a major role in mediating estrogen feedback (Lubahn et al. 1993, Krege et al. 1998).

Progesterone feedback

In the follicular phase in healthy women, administration of progesterone reduced LH pulse frequency, increased the amplitude of LH pulses and decreased mean LH secretion, a pattern of LH secretion that resembles that seen in the mid-luteal phase of the menstrual cycle (Soules et al. 1984). This suggests that progesterone mediates negative feedback on gonadotropin secretion during the luteal phase. PR knock-out mice showed elevated LH levels, although this could be suppressed by progesterone replacement, suggesting multimodal progesterone signalling possibly involving rapid non-genomic progesterone receptor isoforms, PR_A and PR_B (Chappell et al. 1997). Differential negative effect of ovarian steroids on pulsatile LH secretion is observed in sheep, with progesterone preferentially decreasing the frequency and estradiol the amplitude of LH pulses (Goodman and Karsch. 1980). Progesterone actions are estrogen-dependant as the suppressive effects of progesterone were lost in ovariectomised ewes but restored with estrogen replacement (Skinner et al. 1998):

this is analogous to the need for estrogen to induce progesterone receptors and responsiveness in the endometrium. The endogenous opioid dynorphin has also been implicated in mediating progesterone feedback on GnRH/gonadotropin secretion: progesterone increased dynorphin concentrations in the cerebrospinal fluid and upregulated the expression of hypothalamic preprodynorphin mRNA in ovariectomised ewes (Foradori et al. 2005).

A role of progesterone in mediating positive feedback on GnRH/LH secretion has also been described. Progesterone advanced the onset and amplitude of estrogen-induced GnRH/LH surge in ovariectomised rodents and monkeys (Clifton et al. 1975). Furthermore, progesterone antagonist and centrally administered antibodies to progesterone receptor blocked GnRH and LH surge in rats, when measured simultaneously in the median eminence and peripheral blood, suggesting an obligate role of progesterone receptor in the estrogen-induced mid-cycle LH surge (Chappell and Levine. 2000).

Testosterone feedback

Hypogonadal men showed elevated LH pulse frequency and amplitude, both of which were suppressed with exogenous testosterone treatment, suggesting that testosterone or its metabolites exert negative feedback by slowing down pulsatile GnRH secretion (Matsumoto and Bremner. 1984). Similar pattern of LH secretion in response to testosterone were observed in castrated rats (Steiner et al. 1982), rams (D'Occhio et al. 1982) and monkeys (Plant. 1982). Testosterone can be further converted to its metabolite dihydrotestosterone (DHT) by the action of 5α-reductase, and suppression of LH secretion is comparable with both testosterone and DHT (Santen. 1975). Testosterone also undergoes aromatisation to estradiol, but whether this is required for the inhibition of LH secretion in men remains controversial. Santen has argued that since the effects of testosterone and estradiol infusion in healthy men are divergent, with 1) estradiol reducing the amplitude and testosterone the frequency of LH pulses and 2) estradiol blunting and testosterone preserving GnRH sensitivity, aromatisation is not required for hypothalamic negative androgen feedback (Santen. 1975). However, conversion of testosterone to estradiol might be required for the inhibitory effect of testosterone at the pituitary gonadotrope

(Pitteloud et al. 2008). In healthy men, the increase in LH secretion was less than in response to selective estrogen suppression by aromatase inhibitor alone compared to suppression of both estrogen and testosterone by aromatase inhibitor and ketoconazole treatment (Hayes et al. 2001), suggesting an additional action of aromatisation of testosterone to estadiol. To determine whether it is the pituitary or hypothalamic effects of testosterone that require aromatisation, healthy men and those with hypogonadotropic hypogonadism on GnRH therapy were pre-treated with ketoconazole . Whilst testosterone suppressed high LH secretion in healthy men, estradiol and not testosterone decreased LH secretion in patients with hypogonadotropic hypogonadism, indicating that testosterone aromatisation is required for its action at the pituitary (Pitteloud et al. 2008).

1.4.1.2 The site of sex-steroid feedback

Pituitary sex-steroid action

The site of estrogen feedback action was proposed to be the pituitary gland after experiments in rhesus monkeys, where following radio-frequency induced hypothalamic lesions to abolish gonadotropin secretion and responsiveness to estradiol positive feedback, normal ovulatory cycles were restored with intermittent GnRH infusion (Knobil et al. 1980). Similarly, intermittent GnRH administration resulted in ovulation in a patient congenital absence of GnRH (Kallmann syndrome) (Crowley and McArthur. 1980) and in pregnancies in women with hypothalamic amenorrhoea (Leyendecker et al. 1980). In men with idiopathic hypogonadotropic hypogonadism treated with GnRH, administration of estradiol and testosterone decreased LH and FSH secretion, indicating an inhibitory action of gonadal steroids at the level of pituitary (Bagatell et al. 1994). Estrogen induced suppression of LH secretion was also demonstrated from rat gonadotropes in culture (Emons et al. 1986). Estradiol directly inhibits the transcription of FSHβ subunit gene by activating corepressor proteins that bind to the gene (Miller and Miller. 1996). Furthermore, estrogen suppresses FSH secretion indirectly by decreasing activin secretion (Herbison. 1998).

Hypothalamic sex-steroid action

Estrogen feedback has been also demonstrated at the hypothalamic level, suggesting dual-site of sex-steroid feedback in the regulation of gonadotropin secretion. GnRH receptor antagonist suppressed gonadotropin secretion at lower doses during the mid-cycle LH surge than during other phases of the menstrual cycle, suggesting that endogenous GnRH release might be diminished and/or pituitary sensitivity enhanced during the pre-ovulatory gonadotropin surge (Hall et al. 1994). Nevertheless, this is consistent with modulation of GnRH secretion by sex-steroid milieu across the menstrual cycle. The dual-site of estrogen feedback is further supported by administration of the aromatase inhibitor, anastrozole, which in healthy men increased LH pulse frequency suggestive of hypothalamic negative estrogen action, and in men with GnRH-treated hypogonadotropic hypogonadism increased gonadotropin secretion, suggestive of pituitary negative estrogen action (Hayes et al. 2000). Hypothalamic positive estrogenic feedback is demonstrated by studies where estradiol implants in the medial pre-optic region generated an LH surge in rodents (Goodman. 1978). Similarly to estrogen feedback, testosterone also acts directly at the hypothalamus, as in healthy men, GnRH responsiveness on LH secretion was preserved following testosterone infusion (Santen. 1975), and testosterone decreased LH pulsatility following biochemical castration with ketoconazole (Pitteloud et al. 2008). Taken together it appears that in males and females, gonadal steroids mediate gonadotropin secretion by acting at the level of both pituitary and hypothalamus, and this is supported by cognate sex-steroid receptor expression at those locations.

1.4.1.3 KNDy neuropeptides in negative sex-steroid feedback

In the face of GnRH neurones lacking (or expressing rather limited numbers of) sex-steroid receptors, there is compelling evidence to suggest that ERα/PR/AR expressing KNDy neurones constitute this ‘missing link’ as mediators of negative and positive gonadal feedback to the hypothalamic GnRH neurones.

Kisspeptin and neurokinin B of the infundibular/arcuate nucleus have been implicated in mediating negative estrogen action to suppress GnRH and subsequently gonadotropin secretion consistently throughout the species, including humans. In the

infundibular nucleus of postmenopausal women, kisspeptin neurones were hypertrophied, expressed more *KISS1* mRNA and had more autoradiographic grains per neurone than premenopausal women, suggesting that these changes are secondary to the loss of estrogen feedback (Rometo et al. 2007). These hypertrophied neurones were ERα-positive and had upregulated expression of *NKB* mRNA with their neuroanatomical distribution resembling that of kisspeptin neuronal network, suggestive of the synergistic action of kisspeptin and neurokinin B in mediating negative estrogen feedback (Rance et al. 1990, Rance and Young. 1991). Similar adaptations to hypoestrogenic environment are seen in neurokinin B-kisspeptin neuronal network in animal studies. *Kiss*1 mRNA expression was enhanced following an ovariectomy in rodents, sheep and monkeys in the arcuate nucleus but not in more rostral areas, and this was suppressed with estradiol replacement (Smith et al. 2005a, Smith et al. 2005b, Rometo et al. 2007, Smith et al. 2007). Similarly, the expression of neurokinin B increased with ovariectomy and returned to almost undetectable levels with estrogen therapy in the arcuate nucleus in monkeys (Abel et al. 1999, Sandoval-Guzman et al. 2004). Antagonism of kisspeptin receptor abrogated the compensatory LH increase in castrated rodents, suggesting that intact kisspeptin signalling is required for estrogen to mediate its negative effects (Roseweir et al. 2009).

Whilst negative estrogen feedback to reduce GnRH/gonadotropin secretion is mediated by suppressing kisspeptin and neurokinin B activity, the converse, inhibitory opioid component of KNDy system is upregulated. Dynorphin and its cognate κ opioid receptor are expressed by KNDy neurones, and dynorphin neurones co-localise with ERα and PR (Foradori et al. 2002, Foradori et al. 2006, Goodman et al. 2007, Navarro et al. 2009). Naloxone, an opioid receptor antagonist, increased serum LH levels in late follicular and luteal phases of the menstrual cycle (Quigley and Yen. 1980, Shoupe et al. 1985). However, this effect was only apparent in postmenopausal and oophorectomised young women following estrogen or progesterone replacement (Melis et al. 1984, Casper and Alapin-Rubillovitz. 1985, Shoupe et al. 1985). The opioid peptide dynorphin appears to mediate negative progesterone feedback by inducing changes in pulsatile GnRH secretion (Ferin et al.

1984, Karsch. 1987, Goodman et al. 2004). In luteal ewes, low LH pulse frequency (and inferred GnRH pulsatility) was increased with naloxone administration (Goodman et al. 2004).

In contrast to women with menopause, the opioid antagonist naltrexone administered for 6 months increased LH pulse amplitude and plasma gonadal steroids in women with hypothalamic amenorrhoea with subsequent menstruation in some patients (Genazzani et al. 1995b). Lack of response to an opioid antagonist in postmenopausal women and those after oophorectomy may indicate the relative deficiency of dynorphin pathway in mediating negative sex-steroid feedback in the case of hypergonadotropic hypogonadim, in contrast to exaggerated dynorphin signalling contributing to the hypogonadotropic state in those with hypothalamic amenorrhoea. Indeed, the expression of *prodynorphin* mRNA is reduced in the infundibular nucleus in postmenopaual women (Rometo and Rance. 2008) and in the arcuate nucleus of ovariectomised ewes (Foradori et al. 2002, Foradori et al. 2006). It is however possible that there is a threshold maximum to which LH secretion can be induced, accounting for inability of opioid antagonist to further stimulate gonadotropin secretion in women with menopause, which is consistent with relatively small stimulatory effect of KP-10 in this group of women (George et al. 2012).

The situation appears to be distinct in rodents, where despite the co-localisation of KNDy neurones with both ERα and PR, dynorphin does not seem to mediate estrogen negative feedback (Navarro et al. 2009). Estradiol treatment inhibited the expression of dynorphin and kappa opioid receptor in ovariectomised mice, and dynorphin and its receptor knock-out mice had compromised increase in LH secretion following ovariectomy (Navarro et al. 2009). This highlights that although KNDy system is implicated in mediating gonadal feedback to the hypothalamus, species-specific pathways exist, and results from animal studies cannot be directly extrapolated to explain the physiology of KNDy signalling in humans.

AR-expressing KNDy neurones have a role in relaying negative testosterone action. In castrated monkeys, testosterone induced suppression in gonadotropin secretion was accompanied by reduction in the expression of *Kiss1* mRNA in the mediobasal

hypothalamus (Shibata et al. 2007), and this has been demonstrated in lower species (Irwig et al. 2004, Navarro et al. 2004a). Intracerebroventricular administration of kisspeptin antagonist abrogated LH rise associated with orchidectomy in rodents, further implicating the kisspeptin-kisspeptin receptor pathway in hypothalamic testosterone feedback (Roseweir et al. 2009).

1.4.1.4 KNDy neuropeptides in positive sex-steroid feedback

Estrogen feedback switches from negative to positive in the late follicular phase culminating in the GnRH/LH surge necessary for ovulation. However, the neuroendocrine mechanisms involved in this critical event have been unclear. Although negative sex-steroid feedback is mediated by KNDy neurones, their role in positive feedback of gonadal steroids is less consistent and mainly inferred from studies in rodents and sheep, which is further complicated by kisspeptin signalling being site and species specific.

Recent data suggest the role of kisspeptin in mediating positive estrogen feedback in humans. KP-54 (subcutaneous in doses of 1.6 to 12.8nmol/kg) induced LH surge, which was sufficient to trigger oocyte maturation, when administered instead of a conventional human chorionic gonadotropin during an assisted conception cycle, with subsequent live term births reported (Jayasena et al. 2014a). Repeated follicular phase administration of KP-54 (subcutaneous 6.4nmol/kg twice daily for 7 days) shortened the menstrual cycle length in healthy women, indicating advanced LH surge (Jayasena et al. 2013a). The striking positive relationship between estradiol concentration in the late follicular phase and LH response to KP-54 infusion in healthy women lend further support for the involvement of kisspeptin in estrogen feedback (Narayanaswamy et al. 2016a). This is in keeping with data from animal studies. Exogenous kisspeptin administration advanced the LH surge in sheep, whilst antiserum/antagonist to kisspeptin receptor abolished LH peak in rats and sheep (Kinoshita et al. 2005, Caraty et al. 2007, Clarkson et al. 2008, Pineda et al. 2010). Positive feedback actions of estrogen specifically depend on the ERα in kisspeptin neurones, as mice with selective ERα ablation in kisspeptin cells failed to mount LH surges in response to estrogen challenge (Dubois et al. 2015). Although in humans,

ERα is abundantly expressed in kisspeptin neurones (Rance et al. 1990), the role of kisspeptin in physiological positive estrogen feedback in women is less clear.

Hypothalamic kisspeptin population involved in positive estrogen feedback is species specific. In rodents the AVPV nucleus is the location of positive estrogen action, which is not matched in humans, other primates and sheep, where kisspeptin neurones in the infundibular/arcuate relay this function. In rodents, the expression of *Kiss1* mRNA in the AVPV nucleus falls following ovariectomy, but increases after estrogen replacement and is at the peak during proestrus (Smith et al. 2005a, Smith et al. 2006b). Similarly, c-Fos (marker of neuronal activity) expression is at its highest during the LH surge in the AVPV kisspeptin neurones (Smith et al. 2006b). The AVPV nucleus as anatomical site for positive estrogen feedback in rodents is consistent with it receiving afferent fibres from the suprachiasmatic nucleus, the location of circadian clock, which coordinates the timing of LH surge (Khan and Kauffman. 2012). However, in sheep, which, like humans and other primates, have no homologous area to the AVPV nucleus, the expression of *Kiss1* mRNA and c-Fos are dramatically enhanced during the pre-ovulatory LH surge in the arcuate nucleus (Smith et al. 2008b). The above evidence implies that rising levels of estrogen in the late follicular phase exert stimulatory role on kisspeptin secretion in the AVPV nucleus in rodents but the infundibular/arcuate nucleus in sheep and primates, and subsequently culminate in GnRH/LH surge. The reason for kisspeptin mediating positive estrogen feedback via two distinct anatomical sites remains unclear, but may solely reflect species difference.

KNDy neurones of the infundibular/arcuate nucleus may have a role in mediating positive estrogen feedback in some species, including humans. Until very recently, subfertility seen in patients with mutations in NKB/NK3R signalling was the only indirect evidence linking neurokinin B to positive estrogen action (Topaloglu et al. 2009). During the course of preparation of this thesis, a different NK3R antagonist (ESN364) was administered to normal women starting in the follicular phase for 21 days (Fraser et al. 2016). NK3R antagonist delayed LH surge in some women with subsequent delay in luteal progesterone and prolongation of the menstrual cycle length (Fraser et al. 2016). Consistent with the involvement of neurokinin B in the

mid-cycle LH surge, NK3R antagonist abolished LH surge, ovulation and progesterone rise in intact monkeys (Fraser et al. 2015a). However, the mechanism by which neurokinin B antagonism interferes with the mid-cycle LH surge remains unclear as basal LH secretion was unaffected throughout the treatment in both women and female monkeys, and the effect of NK3R antagonist on LH pulsatility was not investigated in this setting (Fraser et al. 2015a, Fraser et al. 2016).

In contrast, central infusion of the NK3R antagonist had no effect on the estrogen-induced LH surge seen in ovariectomised ewes, which suggests that neurokinin B signalling might not be critical for positive estrogen feedback in this animal, further highlighting species difference in the mechanism generating GnRH/LH surge (Li et al. 2015). Unlike kisspeptin, neurokinin B expression did not change in ovariectomised ewes in response to a surge-inducing estrogen challenge (Goubillon et al. 2000) or across the estrous cycle (Li et al. 2015). Nevertheless, KNDy neurone activity is upregulated during the pre-ovulatory LH surge and following estrogen treatment in ewes (Smith et al. 2009a), and NK3R antagonist delayed the onset-to-peak time of LH surge induced by estrogen challenge (Li et al. 2015). In keeping with this observation, NK3R agonist senktide increased LH secretion, resembling 'surge-like' LH levels (Billings et al. 2010). This suggests that neurokinin B pathway is active at the time of estrogen-induced LH surge, but it may not be critical for this physiological event. Accordingly, KNDy neurones are not involved in the pre-ovulatory LH surge in rodents based on their location in the arcuate nucleus only, but not the anteroventral periventricular (AVPV) nucleus, the site for positive estrogen action in this species (Burke et al. 2006, Goodman et al. 2007, Navarro et al. 2009).

Other neurotransmitters may also contribute to kisspeptin-mediated LH surge. Glutamate is described in KNDy neurones and express ERα in the sheep arcuate nucleus, the site of positive estrogen feedback (Oakley et al. 2009). Similarly, tyrosine hydroxylase, galanin, neurotensin, met-enkephalin and cholecystokinin are expressed in the AVPV nucleus, the region specifically implicated in mediating estrogen positive feedback in rodents (Oakley et al. 2009, Porteous et al. 2011).

1.4.2 Endogenous opiates

Endorphins, enkephalins and dynorphins are opioid peptides of their larger precursors acting via G-protein coupled μ, δ and κ opioid receptors. β-endorphin is the major opioid compound secreted by the hypothalamus, which in human brain has been localised to the infundibular nucleus (Wilkes et al. 1980). In healthy men, selective suppression of LH pulse frequency and amplitude by an infusion of DHT and estradiol respectively, was restored by naltrexone (opioid receptor antagonist), suggesting the interaction between the hypothalamic opioid pathway and sex-steroid feedback in the control of GnRH secretion (Veldhuis et al. 1984). The activity of GnRH neurones as inferred by Fos is upregulated following opioid antagonist treatment in the mediobasal hypothalamus in ewes and rams (Boukhliq et al. 1999). The inhibitory opioid tone changes across the menstrual cycle. Whilst naloxone (another opioid receptor antagonist) stimulated LH secretion in the late follicular and luteal phase of menstrual cycle in women, it has no effect in the early follicular phase (Quigley and Yen. 1980). This suggests that endogenous opiates are involved in regulating GnRH/LH secretion when sex-steroid levels are high. Thus opioid input is low during the menses to release the inhibitory break on gonadotropin secretion but is high during the luteal phase to suppress GnRH release. Estrogen-induced LH surges in ovariectomised ewes were delayed by specific μ-opioid receptor agonist (Walsh and Clarke. 1996). Endogenous opiates might mediate negative sex-steroid feedback on gonadotropin secretion. In luteal phase ewes, specific κ-opioid receptor antagonist increased LH pulse frequency, suggesting the role of dynorphin in mediating progesterone negative feedback (Goodman et al. 2004). In postmenopausal and oophrectomised women, naloxone infusion failed to elicit LH secretion unless estrogen, progesterone or both were replaced, suggesting that opioid activity is influenced by sex-steroids and that opioid signalling is diminished post menopause (Casper and Alapin-Rubillovitz. 1985).

Abnormalities in opioid activity have clinical implications. Inhibitory opioid tone might contribute to the hypogonadotropic hypogonadism seen in hypothalamic amenorrhoea since naltrexone treatment restored ovulation in some women (Wildt et al. 1993). Women with hypothalamic amenorrhoea have elevated circulating cortisol

levels, where corticotropin-releasing factor (CRF) might indirectly enhance suppressive opioid effects on GnRH secretion (Suh et al. 1988). Suppression of GnRH in hyperprolactinaemia might be mediated by endogenous opiates. In hyperprolactinaemic rats, low GnRH and LH secretion in the portal blood were associated with elevated levels of β-endorphin, the former of which were increased by naloxone (Sarkar and Yen. 1985). Anovulatory hyperprolactinaemic women were, however, unresponsive to naloxone treatment, but this may reflect deficiency of gonadal steroids since LH secretion increased in ovulatory women with hyperprolactinaemia (Larrea et al. 1995).

1.4.3 Stress and glucocorticoids

Reproductive function is highly sensitive to the situations of stress, including emotional (e.g. depression, restrain), physical (e.g. strenuous exercise), metabolic (e.g. hypoglycaemia) and pathological (e.g. eating disorders, acute and chronic illness), which are associated with transient suppression of pulsatile GnRH secretion and thus quiescence of the pituitary-gonadal axis (Li and O'Byrne. 2015). The pathway by which stressors downregulate the GnRH pulse generator is not entirely clear, but appears to involve numerous neuropeptides, including corticotropin-releasing factor and arginine vasopressin (AVP). There is increasing evidence to suggest that stress neuropeptides mediate their action via kisspeptin signalling.

1.4.3.1 Glucocorticoids

Psychological and physical stress manifest as hypothalamic amenorrhoea in women, where circulating cortisol is elevated and LH pulse frequency is reduced, suggesting that activation of the adrenal axis inhibits GnRH pulse generator (Suh et al. 1988, Berga et al. 1989). Moreover, patients with anorexia nervosa have increased levels of CRF in their cerebrospinal fluid, which is normalised by weight gain (Kaye et al. 1987). Although data is less abundant in men, strenuous military exercising was associated with raised circulating cortisol and suppressed gonadotropin and androgen levels (Opstad. 1992). Similarly, female monkeys exposed to a combination of psychological and physical stressors showed a range of responses, including suppression of gonadotropin secretion, reduced LH pulse frequency and cessation of

ovulatory cycles (Xiao et al. 2002, Bethea et al. 2013). The action of cortisol appears to be through the modulation of pulsatile GnRH secretion as glucocorticoid therapy suppressed GnRH pulse frequency in the hypophyseal portal blood in ewes (Oakley et al. 2009), and in women in the follicular phase LH pulse frequency was reduced (Saketos et al. 1993). It is possible that CRF mediates stress-induced suppression of gonadotropin secretion via kisspeptin signalling, since central CRF infusion in female rats reduced the expression of *Kiss1* and *Kiss1r* mRNA in the pre-optic area and the arcuate nucleus (Kinsey-Jones et al. 2009). This is supported by co-localisation of CRF receptor to kisspeptin neurone in both hypothalamic anatomical sites (Takumi et al. 2012).

1.4.3.2 Inflammation

Acute and chronic illness is not infrequently associated with hypogonadotropic hypogonadism and pubertal delay (van den Berghe et al. 2001). In animal studies, immune/inflammatory challenge on the reproductive system is investigated by the use of lipopolysaccharide (LPS), endotoxin secreted by gram-negative bacteria, which induces acute inflammatory responses resembling those of a bacterial challenge (Iwasa et al. 2008). LPS has been shown to suppress LH pulse frequency in a number of species and GnRH pulsatility in sheep when sampled directly in the hypophyseal portal blood (Battaglia et al. 1998, Xiao et al. 2000). CRF has been implicated in transmitting inhibitory effects of inflammation on reproductive axis since CRF receptor antagonism attenuated LPS-induced suppression of LH secretion in rats and monkeys (Li et al. 2006, Xiao et al. 2007). Furthermore, LPS administration in adult female rats decreased *Kiss1* and *Kiss1r* mRNA expression in the pre-optic area and the arcuate nucleus and this was associated with suppression of pulsatile LH secretion, the latter of which was restored by an injection of kisspeptin (Iwasa et al. 2008, Kinsey-Jones et al. 2009). This suggests the role of kisspeptin in mediating decreased GnRH secretion in response to systemic inflammation.

1.4.3.3 Hypoglycaemia

Insulin-induced hypoglycaemia is known to suppress pulsatile GnRH secretion in rodents, sheep and monkeys (Clarke et al. 1990, Chen et al. 1996, Cates and O'Byrne. 2000). Hypoglycaemic stress also reduced hypothalamic multi unit electrical activity and LH pulses, both of which were restored by glucose, suggesting that glycopenia and not insulin is the causative factor in suppression of the HPG axis (Chen et al. 1992, He et al. 1999). Similarly, in healthy men, LH and testosterone levels deceased in response to hypoglycaemia independent of the dose of insulin infused (Oltmanns et al. 2001). Hypoglycaemia induced suppression of gonadotropin secretion might be mediated via kisspeptin action of the GnRH. In female rats under hypoglycaemic stress, perturbation of pulsatile LH release was associated with downregulation of *Kiss1* and or *Kiss1r* expression in the pre-optic area and the arcuate nucleus (Kinsey-Jones et al. 2009).

1.5 Overall hypothesis

The overarching hypothesis of the work described in this thesis is that pharmacological blockade of neurokinin B signalling will decrease LH secretion and this will be attenuated by exogenous kisspeptin administration, revealing the functional hierarchy between these hypothalamic neuropeptides, whereby neurokinin B signalling is upstream of kisspeptin in modulating pulsatile GnRH secretion. This will be tested in conditions of varying sex-steroid environments, and in states of fast and slow pulsatile LH/GnRH secretion (men, menopausal women, early follicular, late follicular and luteal phases of the menstrual cycle and the mid-cycle LH surge).

Aims

- To determine the physiological role of neurokinin B in men and in different sex-steroid milieu across the menstrual cycle in women;
- To delineate the hierarchical contribution of kisspeptin and neurokinin B in states of slow LH secretion in health;
- To delineate the hierarchical contribution of kisspeptin and neurokinin B in states of high LH secretion in health;
- To determine the role of kisspeptin and neurokinin B in mediating estrogen feedback in a model of the LH surge in women.

Chapter 2. Materials and Methods

2.1 Ethical Approvals and Research Governance

2.1.1 Approvals by ethics committee and local research and development bodies

Clinical studies described in this thesis have been 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 undertaken were basic science proof of concept experiments, involving procedures with human participants and were not clinical trials of an investigational medicinal product (non-CTIMP). Clinical studies undertaking as a part of this thesis fell under the Disorders of Reproductive Endocrine Axis in Man (DREAM) study, which was approved by South East Scotland Research Ethics Committee (Ref: 09/S1101/67) and by NHS Lothian R&D (2010/R/RM/04). The study was sponsored jointly by The University of Edinburgh and NHS Lothian Health Board (Academic and Clinical Central Office for Research and Development, ACCORD). Funding for the study was obtained by a Fellowship awarded from the Wellcome Trust under the Scottish Translational Medicine & Therapeutics Initiative (STMTI) scheme.

2.1.2 Informed consent

All volunteers provided written informed consent. Potential participants received written and oral information, the former being in the form of ethically approved Participant Information Sheets. Oral explanation was provided by the Investigator covering all elements specified in the Participant Information Sheet/Consent form, and the Investigator took informed written consent. All potential participants were given sufficient time to consider the information provided and they were given every opportunity to clarify any points and, if necessary, to seek further information from the Investigator or an independent endocrinologist advisor, details of which were provided in the Information Sheets.

2.1.3 Confidentiality and data protection

All personal information was stored in a password-protected database on the University of Edinburgh computer separate from the main data collection. This

database is accessed by the research team only. Personal information was only used to arrange subsequent visits etc. Participants were given a unique number, which was used to link personal information with study data. All laboratory specimens, evaluation forms, reports, and other study records were link-anonymised to maintain participant confidentiality. Hard copies are held in a locked filling cabinet in a locked room in the Royal Infirmary of Edinburgh, while electronic data are stored on University of Edinburgh servers, which have a number of safety features including encryption. The study was conducted in adherence to NHS Lothian standards for data protection. No portable data storage device was used, except the secure devices provided by NHS. Published results will not contain any personal data that could allow identification of individual participants.

2.1.4 Safety reporting

All adverse events and serious adverse events were recorded by the Investigator. Upon assessment of seriousness, severity, causality and expectedness of any adverse events appropriate actions were taken, and any serious adverse events were reported to ACCORD.

2.2 Recruitment of volunteers

Subjects were recruited for different studies, and included healthy men and women, (both pre- and postmenopausal) (Table 2.1).

2.2.1 Sample size

6-12 healthy human volunteers were recruited into each study. Sample size is based on similar proof of concept mechanistic studies in our lab and by other colleagues, demonstrating statistically significant changes in reproductive hormone concentrations and changes in pulsatile LH secretion (George et al. 2011, Jayasena et al. 2011, George et al. 2012).

2.2.2 Recruitment of healthy men and women

Healthy men and premenopausal and postmenopausal women were recruited by email invitations (approved by research ethics committee) sent within the University of Edinburgh and the NHS Lothian Heath Board. Printed flyers were also displayed within the University of Edinburgh and the clinical areas. Depending on individual preference further information about the study was provided over the telephone, email or printed Participant Information Sheets. Once the potential volunteer was satisfied with the information provided, they were invited to attend for a screening visit. Inclusion and exclusion criteria are shown in Table 2.1.

2.2.3 Screening for eligibility

All subjects attended the Wellcome Trust clinical research facility at the Royal Infirmary of Edinburgh for their screening visit. At this visit informed written consent was signed. If volunteer was eligible based on the history and physical examination, their height, weight, blood pressure and heart rate was recorded and electrocardiogram was performed. Urinary pregnancy test was obtained as appropriate. All volunteers had blood test taken, including full blood count, renal function, electrolytes, liver function, LH, and FSH with addition of estradiol in women and testosterone in men.

Participant group	Inclusion criteria	Exclusion criteria
All volunteers	a) Ability to consent b) Suitable veins for cannulation and/or repeated venepuncture	a) Undiagnosed structural diseases/ previous surgery or radiation affecting hypothalamus or pituitary; b) Clinically significant cardiac, hepatic, renal, pulmonary, endocrine, neoplastic abnormalities; c) Uncontrolled hypertension or diabetes; d) Pregnancy/lactation; e) Concomitant use of cytochrome P450 inducers or inhibitors within 4 weeks of the screening visit;
Healthy men	a) Aged 18-45; b) Normal pubertal development;	
Healthy premenopausal women	a) Aged 18-45; b) Normal pubertal development; c) Regular menstrual cycle (25-35 days); d) Using appropriate non-hormonal contraception, including abstinence, condoms, sterilisation, vasectomy;	a) Hysterectomy b) Bilateral or unilateral oophorectomy; c) Concomitant use of hormonal contraceptives within 8 weeks or antiandrogenic drugs within 12 weeks of the screening visit;
Healthy postmenopausal women	a) At least 12 months of amenorrhoea; b) FSH level above upper limit and estradiol level below lower limit of laboratory reference range;	a) Taking HRT b) Taking aromatase inhibitors or selective estrogen receptor modulators

Table 2.1 Subjects recruited into different studies, and inclusion and exclusion criteria used

2.3 Hormones and drugs

2.3.1 Kisspeptin-10

Human Kisspeptin-10 (KP-10) was custom synthesized under GMP standards (Bachem GmBH, Weil am Rhein, Germany). 1 mg Kisspeptin-10 was dissolved in 5 ml sterile normal (0.9%) saline immediately before administration.

In healthy men and postmenopausal studies, Kisspeptin-10 was administered by an intravenous bolus at 0.3 μg/kg. The dose of KP-10 was selected based on a previous study administering KP-10 in healthy male volunteers (George et al. 2011). KP-10 at 0.3 μg/kg was the lowest dose to cause maximal stimulation in LH secretion with no evidence of tachyphylaxis.

In a model of the LH surge in healthy premenopausal women, KP-10 was administered as an intravenous infusion for 7 hours at 4 μg/kg/hour.

2.3.2 NK3 receptor antagonist (AZD4901)

The specific neurokinin-3 receptor (NK3R) inhibitor AZD4901, formulated as 20 mg tablets, was gifted by AstraZeneca, UK. In all studies, AZD4901 was administered as 40 mg oral twice daily for 7 days.

The dose of 40 mg twice daily was chosen as this is the highest yet administered to healthy volunteers, and showed efficacy in reducing LH secretion and LH pulse frequency in women with PCOS (George et al. 2016).

2.3.3 Transdermal estradiol

Two transdermal Evorel patches releasing 100 μg 17β estradiol each per 24 hours (Janssen-Cilag Buckinghamshire, UK) were used as exogenous estradiol treatment.

2.3.4 Vehicle

Sterile normal (0.9%) saline (Baxter Healthcare Ltd, Thetford Norfolk, UK) was used as vehicle.

2.4 Devices and imaging tools

2.4.1 Intravenous infusion devices

The syringe (20 ml BD Plastipak, Spain) and line (150 cm Sae-flo MD, Wescott Medical Limited, Durham) for infusion were first coated for 30 min with Kisspeptin-10 to minimise peptide loss from adherence to the plastic. Infusion pumps (Alaris GH syringe pump, Cardinal Health, Rolle, Switzerland) designed to deliver low infusion rates were used for Kisspeptin-10 administration.

2.4.2 Ultrasonography

Transvaginal ultrasound scan (Xario 200, Toshiba, The Netherlands, 7.5MHZ frequency) was performed to measure largest follicle (mm), any follicles of ≥10 mm diameter, endometrial thickness and appearance of corpus luteum. The Investigator performed all ultrasound scans.

2.5 Collection of samples

2.5.1 Peripheral blood

For single time point peripheral blood sampling, venepuncture was performed using green 21-gage butterfly needle (Sarstedt UK Ltd, Leicestershire). For infusion studies, blood samples were collected via an indwelling intravenous cannula 17-gauge (B. Braun, Melsungen, Germany) inserted into the antecubital fossa under aseptic technique. After each samples drawn, 2 mls of normal saline was used to flush the cannula to prevent coagulation within the sampling system. Before each sample drawn, 2 mls of blood was discarded to avoid dilution of the sample from residual saline in the cannula.

Samples for hormone analysis (LH, FSH, estradiol, inhibin B, progesterone and testosterone) were collected into vacuum serum tubes (S-Monovette, Sarstedt UK Ltd, Leicestershire). They were processed at the Clinical Research Facility by centrifugation at 4°C for 10 min at 3000 rpm. Serum was then separated into pre-labelled aliquot tubes and frozen at -20°C or below until analysis. Safety samples (full blood count, renal function, electrolytes and liver function) were collected into vacuum serum tubes (S-Monovette, Sarstedt UK Ltd, Leicestershire) and processed by the NHS Lothian laboratory at the Royal Infirmary of Edinburgh.

2.5.2 Urine

Urine samples for progesterone analysis were collected by participants into 7 ml bijous (Greiner Bio One, Stonehouse, UK) and frozen in a domestic freezer prior to batch transfer for storage in dedicated freezers at -20°C or below at the Queen's Medical Research Institute until analysis.

2.6 Analysis of samples

LH, FSH, estradiol, inhibin B, progesterone and testosterone measurement

Serum and urinary hormone assays were carried out by Dr Forbes Howie at the MRC Centre for Reproductive Health core immunoassay laboratory, except testosterone, which was assayed in the NHS Lothian laboratory at the Royal Infirmary of Edinburgh.

LH and FSH concentrations were measured by an in-house ELISA as previously described (George et al. 2011). The inter-assay and intra-assays coefficient of variation (CV) for LH and FSH were <10%. Lower detection limit for LH was 0.2 IU/l and for FSH 0.05 IU/l.

17β-estradiol was performed on a Roche Cobas E411 immunoassay analyser (Roche Diagnostics, Burgess Hill, UK) using the manufacturer's kits and controls according to their instructions. The lower limit of detection was 18.4 pmol/l. The inter-assay CV was found to be <5% and intra-assays CV was 6.5%.

The Inhibin B ELISA was an enzymatically amplified three-step sandwich assay from Beckman Coulter Inc. Brae, CA, USA. According to the manufacturer, the lowest amount of inhibin B that could be detected with a 95% probability is 2.6 pg/ml. The inter-assay CV was <8% across the dynamic range of 10 to 950 pg/ml. Insufficient assays were performed to calculate an inter-assay CV but the manufacturer claim it is in the region of 6% at 100 pg/ml. All samples were analysed in one batch.

Testosterone was measured by liquid chromatography-tandem mass spectrometry by NHS Lothian clinical laboratory according to their standard operating procedures.

Lower limit of detection was 0.3 nmol/l. The inter-assay and intra-assays CV were approximately 12%.

Progesterone was measured by an in-house ELISA. The inter-assay CV for low and high progesterone pools respectively were 11.4 and 9.1% and the intra-assay CV were 8.9 and 5.6%. The lower limit of detection was calculated at 0.1 ng/ml.

Urinary progesterone concentrations were expressed as a ratio of the creatinine concentration measured colorimetrically. Creatinine was determined utilising a commercial kit (Alpha Laboratories Ltd. Eastleigh, UK) adapted for use on a Cobas Fara centrifugal analyser (Roche Diagnostics Ltd, Welwyn Garden City, UK). Within run precision was CV < 3% while intra-batch precision was CV < 5%.

All samples from each of the study visits were analysed together in one batch, in duplicate.

LH pulsatility measurement

Analysis of pulsatile LH secretion was kindly performed by Prof Johannes Veldhuis at the Endocrine Research Unit at the Mayo Clinic (Rochester USA). Analysis was performed blinded to treatment allocation. The number of LH pulses, secretory mass of LH per pulse, basal (nonpulsatile) and pulsatile (integral of dual amplitude and frequency regulation) LH secretion was identified by an established deconvolutional algorithm with cluster analysis (93% sensitivity and specificity) (Veldhuis et al. 2008, Liu et al. 2009). ApEn was quantified as a measure of secretory regularity.

Screening and safety bloods

Full blood count, renal function, electrolytes and liver function tests were processed by the NHS Lothian clinical laboratory at the Royal Infirmary of Edinburgh according to their standard operating procedures. Results were reported on the computerised NHS Lothian TRAK system.

2.7 Statistical analysis

Hormone data not normally distributed were log-transformed prior to statistical analysis, resulting in a distribution that approximated a normal distribution. Data were assessed for Gaussian distribution by Shapiro-Wilk normality test (for data points ≥7) or Kolmogorov-Smirnov test as appropriate (for data points <7).

Mean and area under the curve (AUC) hormone concentration comparisons over time and between treatments were performed using analysis of variance (ANOVA) with repeated measures as appropriate followed by post hoc analysis with Bonferroni's correction for multiple comparisons. Peak hormone concentrations and their peak menstrual cycle day were compared by Student's paired t-test or Wilcoxon matched-pairs signed rank test. The null hypothesis of equal variation in cycle days was tested by Levene's test for homogeneity of variance. Area under the curve for hormone concentrations during 6-8 hours of frequent sampling was calculated by trapezoid integration.

The relationship between the timing of peak hormone concentration and treatment was assessed by chi-square test. Pearson correlation coefficient was computed to assess the relationship between estradiol concentrations and LH response to KP-10.

Paired Student's t-test (for parametric data) or Wilcoxon matched-pairs signed rank test (for non-parametric data) was used to assess changes in LH pulsatility, as appropriate.

Data on follicle diameters and endometrial thickness were compared over time and between treatments using ANOVA with repeated measures as appropriate followed by post hoc analysis with Bonferroni's correction for multiple comparisons.

Additional expert input in statistical analysis was kindly provided by Cat Graham.

Data are presented as mean ± SEM. Differences were regarded as significant at a two-sided $p<0.05$. The statistical software package GraphPad Prism (GraphPad, San Diego, California) was used for data analysis.

Chapter 3. Neurokinin B and Kisspeptin in the regulation of gonadotropin and testosterone secretion in healthy men

3.1 Introduction

Kisspeptin and neurokinin B (NKB) are hypothalamic neuropeptides now recognised as being key regulators of pulsatile GnRH secretion and thus central to the control of the human reproduction (Skorupskaite et al. 2014). Men and women with loss-of-function mutations in kisspeptin, neurokinin B or their cognate receptors (KISS1R and NK3R) show reduced GnRH secretion and subsequently hypogonadotropic pubertal delay (de Roux et al. 2003, Seminara et al. 2003, Topaloglu et al. 2009, Topaloglu et al. 2012), whilst activating mutations in kisspeptin receptor are associated with precocious puberty (Teles et al. 2008).

Whilst kisspeptin potently stimulates gonadotropin secretion (Dhillo et al. 2005, Chan et al. 2011, George et al. 2011, Jayasena et al. 2011, Jayasena et al. 2015a) and increases LH pulsatility in men (George et al. 2011), effects of exogenous neurokinin B administration are discordant. In animal studies both stimulatory and inhibitory actions of neurokinin B on LH secretion has been observed (Billings et al. 2010, Navarro et al. 2011a, Ramaswamy et al. 2011), whilst it had no effect on either reproductive hormone concentrations, nor LH pulsatility when infused for 8 hours in healthy men and women (Jayasena et al. 2014c). During the preparation of this thesis, NK3R antagonist ESN364 was administrated to human volunteers (Fraser et al. 2016). In women, there was no change in baseline gonadotropin secretion throughout the 21 day follicular phase administration of NK3R antagonist, but a delay in the LH surge was observed, likely secondary to a delay in the pre-ovulatory estradiol rise (Fraser et al. 2016). These findings are similar to those in intact adult female monkeys receiving the same NK3R antagonist (Fraser et al. 2015a). In men, NK3R antagonist suppressed LH and testosterone secretion, but not FSH (Fraser et al. 2016). However, this effect was temporary lasting only few hours post dosing with hormones returning to baseline within 24 hours and concentrations being no different after 10 days of treatment compared to pre-treatment values (Fraser et al.

2016). Antagonism of NK3R has been shown to decrease LH secretion and reduce the frequency of LH pulses in states of high LH output, such as in women with PCOS (George et al. 2016) and gonadectomised animals (Fraser et al. 2015a, Li et al. 2015), indicating a role of neurokinin B in the regulation of pulsatile GnRH and thus LH secretion. However, the inhibitory action of neurokinin B antagonism on GnRH/LH pulsatility has not been investigated in states of slow LH secretion and in health.

Kisspeptin and NKB are co-expressed by some neurones within the hypothalamus, in humans and other species, which also co-express the opioid dynorphin and have been termed KNDy neurones (Cheng et al. 2010). The interaction and functional hierarchy between kisspeptin and neurokinin B is largely inferred from studies in animal models. *Kissr1* knockout mice were unable to mount a stimulatory effect of NK3R agonist senktide on LH secretion (Garcia-Galiano et al. 2012), and in juvenile male monkeys, KP-10 stimulated LH secretion after pre-treatment with the NK3R antagonist (Ramaswamy et al. 2011), suggesting the action of neurokinin B to be upstream of kisspeptin. In patients with inactivating genetic defects in NKB (*TAC3*) and its receptor (*TACR3*), showing slow GnRH secretion as inferred from low LH secretion (mean 0.1 pulses per hours) and near normal FSH, exogenous KP-10 restored LH pulse frequency to normal (Young et al. 2013), providing further evidence that neurokinin B is functionally upstream of kisspeptin in the regulation of GnRH secretion. However, this is the only human study investigating the hierarchy of kisspeptin and neurokinin B and there are no studies looking at this in a physiological rather than disease model.

The antagonistic effect of neurokinin B and its interaction with kisspeptin in the regulation of the male reproductive axis, a state of slow LH secretion, was therefore explored by the use of a specific NK3R antagonist.

3.2 Objectives

The objectives of the study were, in healthy men:

1) To determine the effect of NK3R antagonist on LH, FSH and testosterone secretion;
2) To investigate the effect of NK3R antagonist on LH pulsatility;
3) To determine if KP-10 stimulated LH secretion is abrogated in the presence of NK3R antagonist, thereby revealing the functional hierarchy of neurokinin B being proximal to kisspeptin.

3.3 Methods

3.3.1 Participants

Six healthy men, aged 23-39 years were recruited to this study; all volunteers provided informed written consent. Subjects were eligible based on their inclusion and exclusion criteria (Table 2.1 Inclusion and exclusion criteria, Chapter 2), had normal physical examination, and full blood count, renal function, electrolytes, liver function and electrocardiogram were within normal limits.

3.3.2 Study drugs

The specific neurokinin-3 receptor (NK3R) inhibitor AZD4901 was administered orally at 40 mg twice daily. Kisspeptin-10 was administered by an intravenous bolus at 0.3 μg/kg and prepared as described in Chapter 2 (2.3.1 Kisspeptin-10).

3.3.3 Protocol

Investigation of the effect of NK3R antagonism on gonadotropin and testosterone secretion.

A schematic representation of the protocol is shown in Figure 3.1. Volunteers were administered the NK3R antagonist for 7 days. Peripheral venous blood was sampled for spot LH, FSH and testosterone 24 hours before treatment (pre-treatment) and on days 2, 4, 6 and 7 of NK3R antagonist administration. Once daily blood sampling was performed in the morning: during treatment, this was immediately prior to the next dose of NK3R antagonist, i.e. 12 hours after the previous dose.

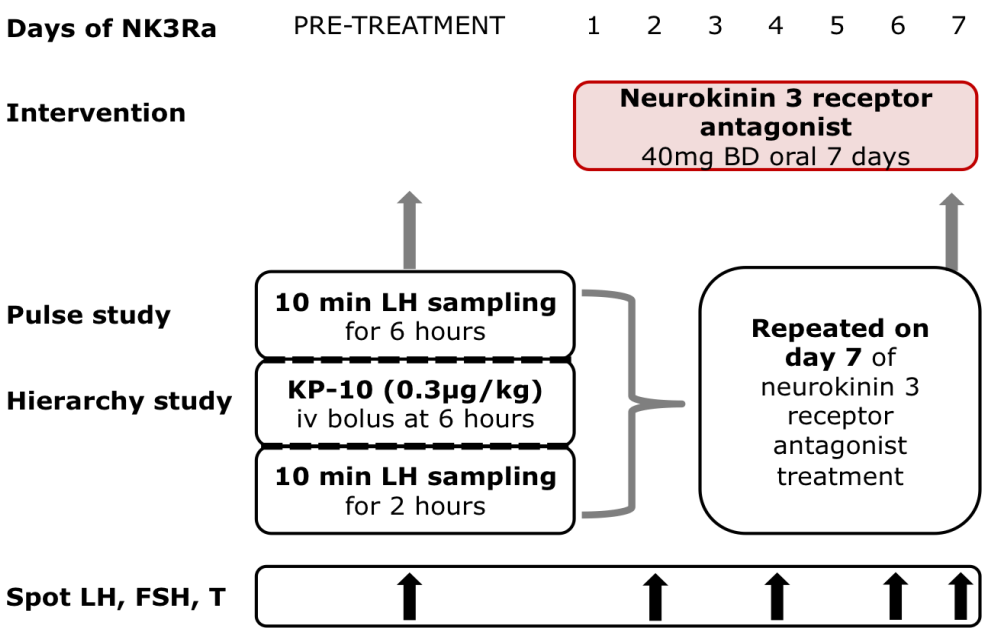


Figure 3.1 Study protocol.

6 healthy men were administered NK3R antagonist AZD4901 orally for 7 days. LH, FSH and testosterone were measured throughout the study. LH pulsatility was assessed during 10 minute blood sampling for 6 hours on the day before and on the last day of NK3R antagonist treatment. KP-10 was administered by intravenous bolus at 6 hours with further frequent blood sampling for 2 hours.

Investigation of the effect of NK3R antagonist on LH pulsatility.

On the pre-treatment day and on the last day of NK3R antagonist administration (day 7) volunteers attended our clinical research facility for 8 hours. All visits commenced between 0800 and 0900 hours to avoid diurnal variation. Blood samples were collected via an indwelling intravenous cannula at 10 min intervals for 6 hours for assessment of LH pulsatility. The dose of NK3R antagonist was administered immediately prior to the start of sample collection (day 7).

Establishment of the hierarchy between kisspeptin and neurokinin B on LH secretion.

Kisspeptin-10 was administered at 6 hours of the pulsatility study described above with further 10 min blood sampling for 2 hours after administration.

Safety profile. Safety blood tests including full blood count, renal function and electrolytes, and liver function were checked before commencing the NK3R antagonist, at the end of each 8 hour visit for frequent blood sampling and 2-3 weeks later.

3.3.4 Analytical methods

Hormone assays, pulsatile LH secretion and safety blood tests were analysed as described in Chapter 2.

3.3.5 Statistical analysis

Mean LH, FSH and testosterone concentrations over time were compared using one-way analysis of variance (ANOVA) with repeated-measures followed by Bonferroni's post hoc correction for multiple comparisons. Area under the curve (AUC) of LH for each of the 8 hours of frequent sampling was determined by trapezoid integration on the pre-treatment day (control) and on day 7 of NK3R antagonist administration, and change in AUC LH during one hour pre KP-10 injection and for 2 consecutive hours post KP-10 injection was calculated. Comparisons across both time and between treatments were performed using repeated-measures two-way ANOVA with Bonferroni's multiple comparisons post hoc analysis. Paired Student's t-test (for normally distributed data: basal and pulsatile LH secretion, mass per pulse, ApEn) or Wilcoxon matched-pairs signed rank test (for data that did not have a normal distribution: pulse frequency) was used to assess changes in pulsatile nature of LH secretion. Data are presented as mean ± SEM.

3.4 Results

3.4.1 NK3R antagonist decreases gonadotropin and testosterone secretion

LH secretion decreased with NK3R antagonist administration ($p<0.03$), demonstrating a biphasic response: LH levels fell after 24 hours of treatment (4.5±0.6 IU/l pre-treatment to 1.7±0.2 IU/l day 2, $p<0.05$), then recovered (4.2±0.7 IU/l day 4 and 3.7±0.7 IU/l day 6) but was again decreased on day 7 (2.5±0.6 IU/l vs pre-treatment, $p<0.05$) (Fig. 3.2A). To detect subtle changes in hormone secretion over time potentially overlooked by performing single time spot blood sampling, a more detailed analysis of LH secretion every 10 minutes for 6 hours post NK3R antagonist dose showed a decrease in AUC LH secretion throughout the 6 hour-period on the last day of NK3R antagonist administration compared to pre-treatment (16.8±2.7 vs 8.7±1.5 IU/l over 6 hours with NK3Ra, $p=0.0007$, Fig. 3.2B).

FSH secretion was also suppressed during NK3R antagonist administration ($p=0.001$), being significantly lower after 24 hours of treatment (3.6±0.3 vs 2.4±0.4 IU/l, $p<0.001$) (Fig. 3.2C). FSH showed a comparable pattern to changes in LH over the following days, being significantly lower than pre-treatment on days 4 and 7 (each $p<0.05$ vs pre-treatment) but not on day 6 of taking NK3R antagonist administration.

Testosterone secretion declined rapidly in response to NK3R antagonist at 24 hours (18.4±1.6 pre-treatment vs 5.6±1.5 nmol/l, $p<0.01$) and was consistently suppressed for the remainder of the treatment period ($p<0.05$ day 4, 6 and 7 vs pre-treatment) (Fig. 3.2D). Serum testosterone recovered to pre-treatment in all subjects 2 weeks later (19.8±2.0 nmol/l).

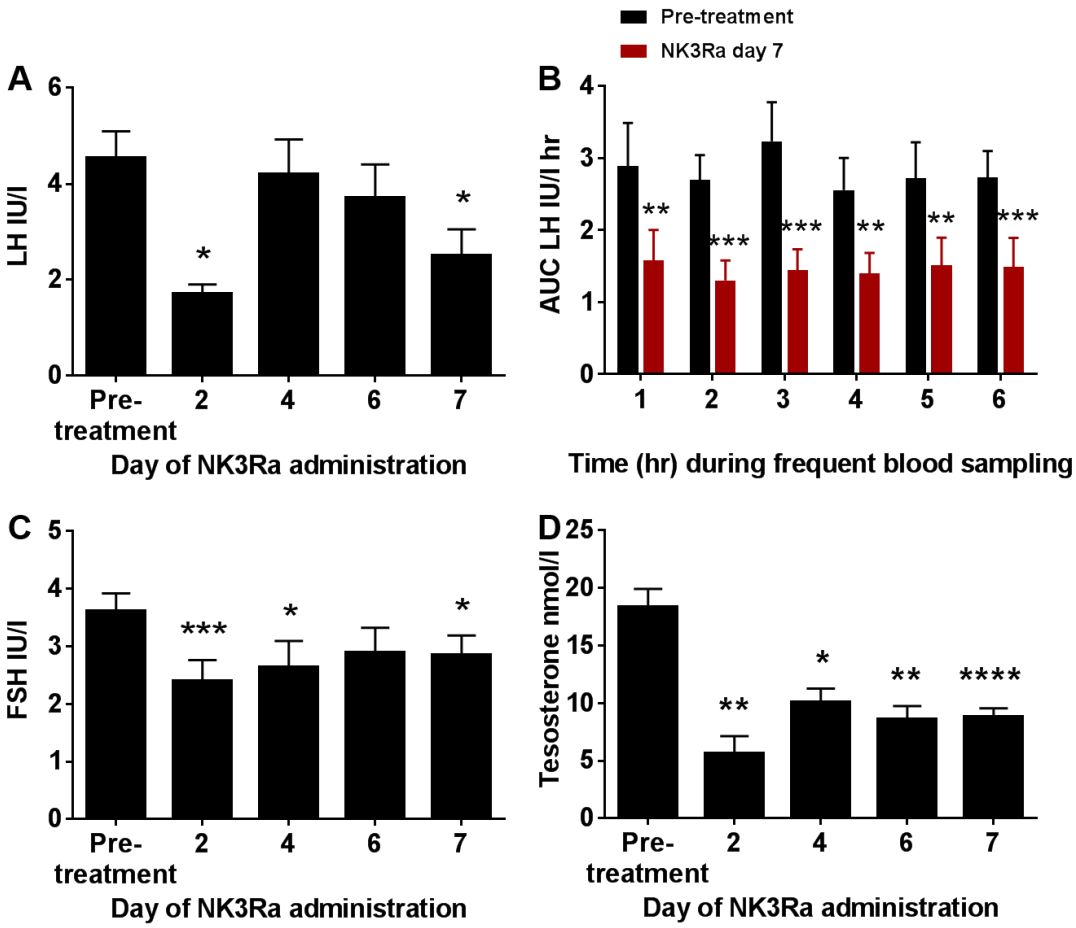


Figure 3.2 LH, FSH and testosterone response to administration of NK3R antagonist in healthy men.

(A) Mean LH concentrations during 7 days of NK3R antagonist treatment and **(B)** AUC LH during 6 hours of 10 minute LH sampling pre-treatment and on day 7 of NK3R antagonist administration (n=6). Mean FSH **(C)** and testosterone **(D)** response to NK3R antagonist administration (n=6). Data are presented as mean ± SEM. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$ vs pre-treatment.

3.4.2 Effect of NK3R antagonist on pulsatile LH secretion in men

LH pulse profile from all individual subjects before and after 7 days of NK3R antagonist treatment is shown in figure 3.3. The LH pulse frequency was unchanged by NK3R antagonist (0.50±0.09 vs 0.47±0.07 pulses/hr, ns) (Fig. 3.4A). However consistent with suppressed LH secretion, other parameters of LH pulsatility were reduced. Both basal (nonpulsatile) and pulsatile mass of LH secretion were lower with NK3R antagonist treatment compared with pre-treatment day (Fig. 3.4 B-C, both <0.02). The secretory mass of LH per pulse (Fig. 3.4D) was not significantly changed. NK3R antagonist improved the orderliness of LH secretion pattern as assessed by approximate entopy (ApEn) (p=0.02) (Fig. 3.4E). ApEn is a statistical measure of the pattern of pulsatile LH secretion and not something that can be illustrated graphically.

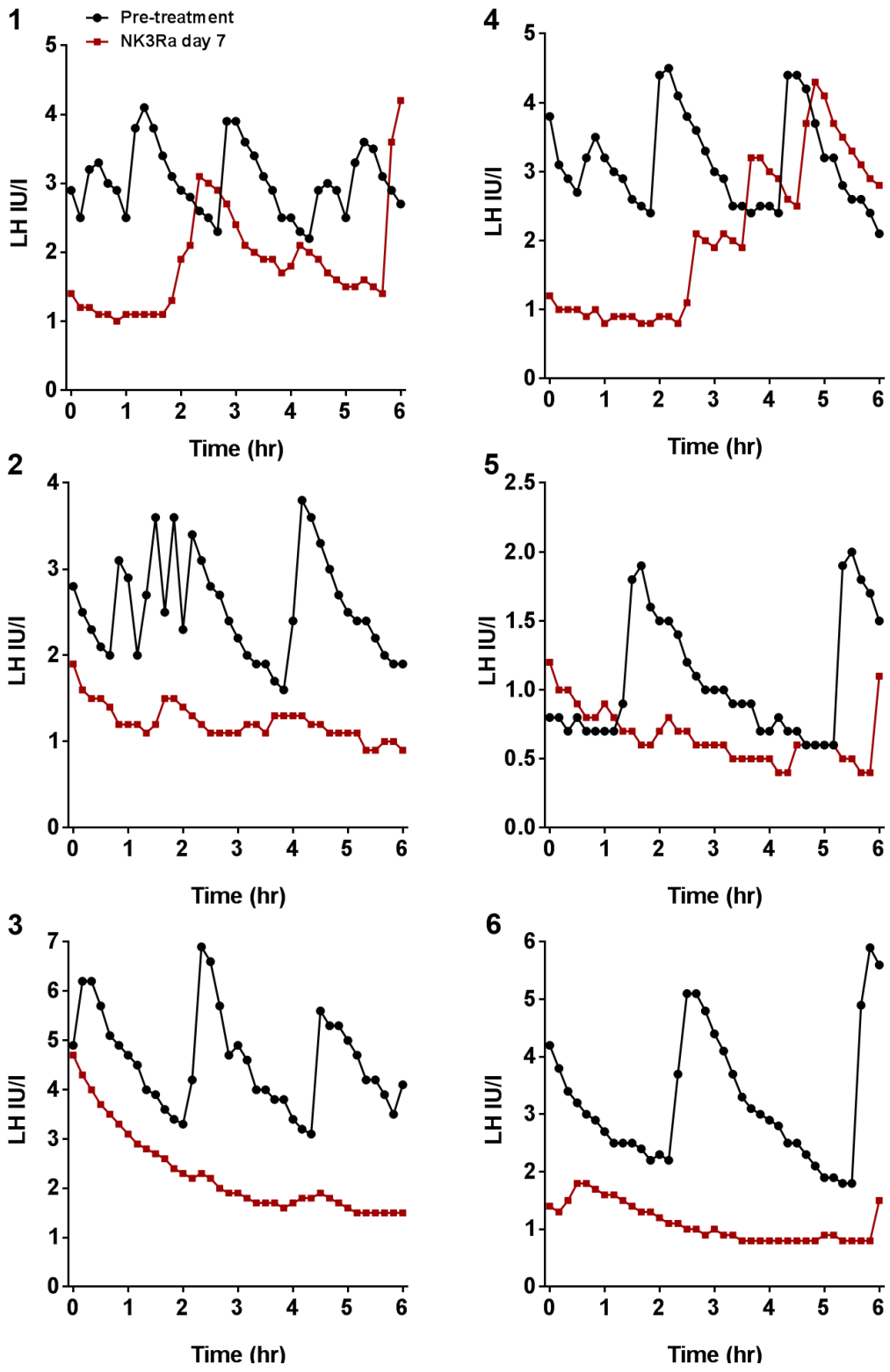


Figure 3.3 Individual LH pulse profile.

LH profiles sampled at 10 min intervals for 6 hours from 6 individual subjects **(1-6)** pre-treatment (*black circles*) and on day 7 of NK3Ra administration (*red squares*).

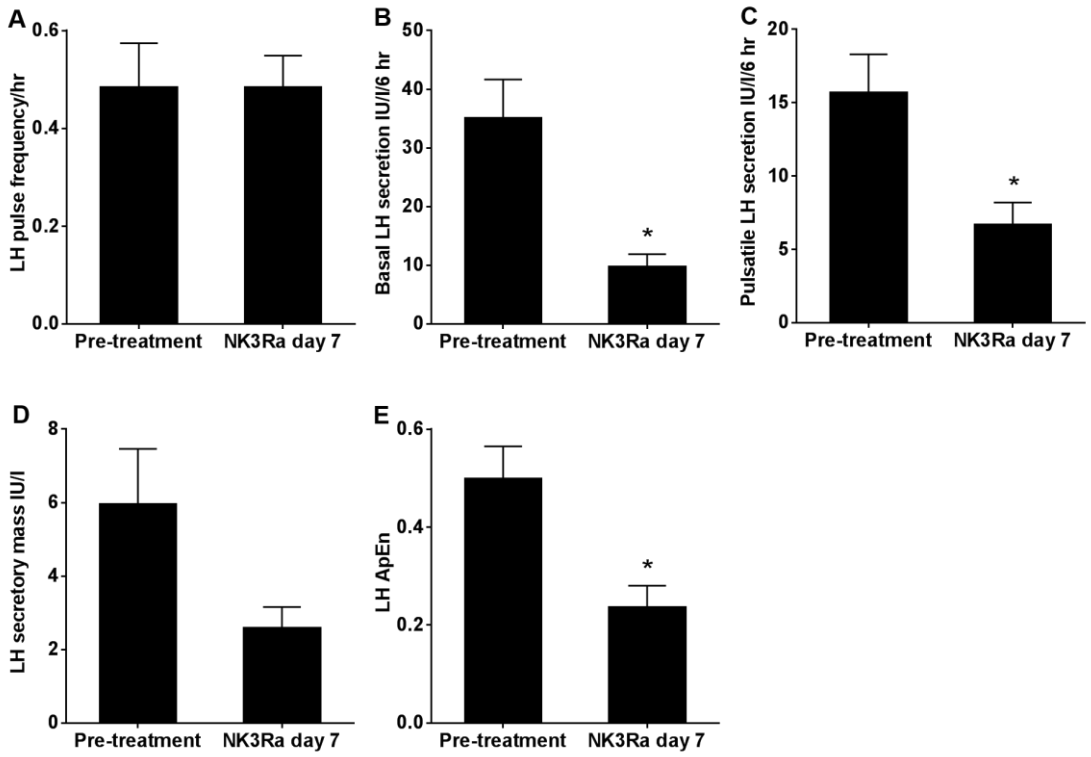


Figure 3.4 Analysis of 6 hour LH secretory pattern on day 7 of NK3R antagonist treatment compared to control pre-treatment day in healthy men.

Mean LH pulse frequency **(A)**, basal (nonpulsatile) LH secretion **(B)**, pulsatile LH secretion **(C)**, secretory mass of LH per pulse **(D)** and the relative orderliness/regularity of LH secretory pattern **(E)** on day 7 of NK3R antagonist treatment compared to pre-treatment day. Data are presented as mean ± SEM. *, $p<0.05$.

3.4.3 NK3R antagonist does not reduce kisspeptin-induced LH secretion

To assess the hierarchical interaction between kisspeptin and neurokinin B in the regulation of LH secretion in men, an intravenous injection of kisspeptin-10 was administrated after 6 hours of blood sampling on pre-treatment day and on the last day of NK3R antagonist treatment (Fig. 3.5). KP-10 elicited a rapid increase in LH secretion (3.0±0.6 at 6 hours vs 4.8±0.5 IU/L at 7 hours, $p<0.05$ and AUC LH 2.7±0.4 6 hours vs 5.2±0.5 IU/l hr 7 hours, $p<0.01$) (Fig. 3.5 A and B). The stimulation of LH secretion by KP-10 persisted in the presence of NK3R antagonist (mean LH and AUC LH for KP-10+NK3R antagonist: 6 hours vs 7 and 8 hours, all $p<0.05$; Fig. 3.5 A and B) and was similar to stimulated LH secretion after KP-10 alone (AUC LH 7 and 8 hours KP-10: 5.2±0.5 and 3.9±0.4 vs 5.2±0.7 and 4.0±0.6 IU/l hr with NK3R antagonist, ns). However, calculation of the LH response to KP-10 as ΔAUC between hours 6 and 7/8 suggested that it was enhanced during NK3R antagonist treatment (KP-10 alone ΔAUC LH 7 and 8 hours: 2.5±0.2 and 1.2±0.2 vs 3.8±0.5 and 2.5±0.4, KP-10 with NK3R antagonist, both $p<0.05$) (Fig.3.5C). This may reflect the lower LH concentrations with the NK3R antagonist at the time of KP-10 administration with similar LH concentrations after KP-10 injection.

3.4.4 Tolerability and safety

NK3R antagonist treatment was well tolerated. One man reported reduced libido whist on NK3R antagonist and this recovered after the completion of the study. Haematology and biochemistry safety parameters remained stable in all subjects throughout the study period.

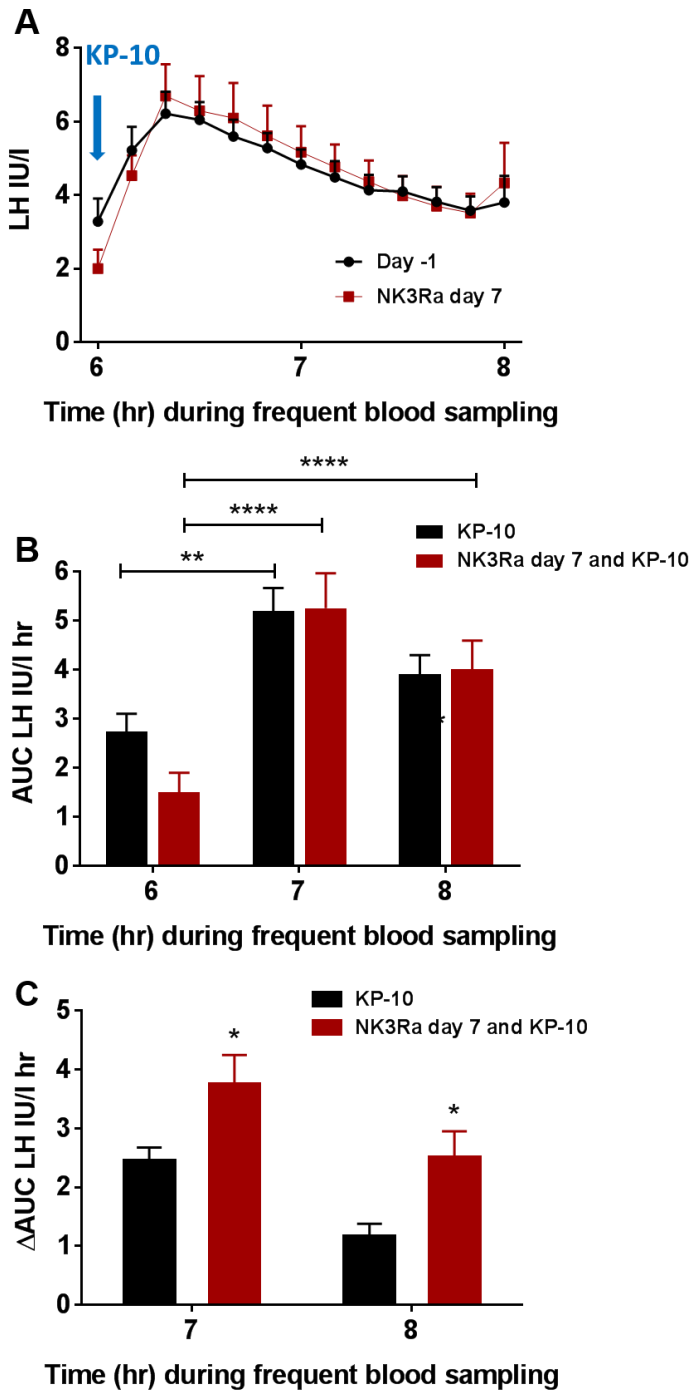


Figure 3.5 LH response to KP-10 injection with and without 7 days of NK3R antagonist administration in healthy men.

(A) Mean LH concentrations from 6 hours of frequent sampling; **(B)** AUC LH and **(C)** ΔAUC LH were compared over one hour (hour 6) before KP-10 administration, with 2 hours post KP-10 administration (7 and 8 hours), with and without NK3R antagonist treatment during frequent blood sampling for LH every 10 minutes in the same 6 healthy men. Mean ± SEM. *, $p<0.05$; **, $p<0.01$; ****, $p<0.0001$.

3.5 Discussion

In the present study the role of neurokinin B and its interaction with kisspeptin in the regulation of GnRH and LH/FSH secretion was investigated in healthy men, where LH pulsatility is characteristically slow. Pharmacological blockade of NKB-NK3R signalling caused a rapid and marked decrease LH, FSH and testosterone secretion, although with a complex pattern of change over the week of treatment, whilst the stimulatory LH response to kisspeptin was maintained after 7 days of NK3R antagonist. NK3R antagonism did not affect the frequency of LH pulses in men, but other aspects of the pulsatile nature of LH secretion were markedly reduced. Taken together, these data show that NKB is important in the physiological regulation of GnRH/LH and testosterone secretion in men. They support a predominantly hierarchical relationship whereby the action of neurokinin B is proximal to kisspeptin in the modulation of GnRH/LH secretion, as previously indicated in men with NKB/NK3R mutations (Young et al. 2013). However, the pulse analysis suggests that the main effect of NKB on GnRH is through an overall reduction in both basal and pulsatile secretion without a change in pulse frequency, in contrast to the only previous data from women with PCOS, where an impact on pulse frequency was identified (George et al. 2016).

These data demonstrate that in men, NK3R antagonist decreased LH secretion in a biphasic pattern, with significant reductions after 24 hours (day 2) and on day 7. Detailed 10-minute assessment showed suppression in LH secretion persisting for the duration of 6 hour sampling on day 7 of NK3R antagonist administration. Consistent with this being by suppression of GnRH secretion, NK3R antagonist also lowered FSH concentrations on day 2, 4 and 7 of treatment, albeit to a lesser extent than with LH secretion. FSH secretion is less sensitive to rapid changes in GnRH secretion, which may account for the slightly different profile of the FSH response to NK3R antagonism over the 7 days of treatment. A different NK3R antagonist, ESN364, suppressed LH secretion in ovariectomised ewe and castrate monkeys (Fraser et al. 2015a, Li et al. 2015) but with only a transient 3 hour effect in normal men (Fraser et al. 2016) and did not affect FSH secretion in either men (Fraser et al. 2016) or castrate monkeys (Fraser et al. 2015a). There were no differences in reproductive

hormone concentrations on day 10 of ESN364 administration when compared to pre-treatment levels in men (Fraser et al. 2016) and similarly, no change in LH secretion was observed with follicular phase administration of this NK3R antagonist in normal women or in intact female monkeys, although the LH surge was delayed (Fraser et al. 2015a, Fraser et al. 2016). This may reflect the greater effect on LH (and by inference GnRH) observed with NK3R antagonist AZD4901 used in our studies. Furthermore, Fraser and colleagues did not assess the effect of NK3R antagonist on pulsatile LH secretion to determine the nature of its effect on GnRH secretion.

Sustained suppression of testosterone secretion was observed throughout the 7 days of treatment. Hitherto, this NK3R antagonist has been administered in states of high LH output and was shown to reduce both LH and testosterone secretion in women with PCOS (George et al. 2016). Although recovery of suppressed LH secretion with low testosterone levels on day 4 and 6 in the present study may indicate a direct gonadal effect of NK3R antagonist, and *NKB-NK3R* mRNA has been detected in human granulosa cells (Cejudo Roman et al. 2012, Garcia-Ortega et al. 2014), there are no data on this in human testes. The simultaneous decrease in serum LH and testosterone concentrations within 24 hours of treatment suggests a central role of neurokinin B in the regulation of GnRH/LH secretion, which is further supported by changes in the pattern of LH pulsatility. It seems likely that the initial fall in LH and testosterone levels caused a perturbation in testosterone negative feedback with a secondary recovery in LH secretion, with the suppressive effect re-established by the end of the week of treatment. It remains to be determined if this suppression would persist with prolonged antagonism of NKB-NK3R signalling.

The present study has shown for the first time that in normal men NK3R antagonist markedly reduced both basal i.e. nonpulsatile LH secretion and total amount of LH secreted in pulses, and the secretory mass of LH per pulses also trended downwards, contributing to overall lower serum LH concentrations by the end of the treatment. The regularity of LH secretory pattern and by inference GnRH secretion showed greater orderliness as indicated by reduced ApEn. In contrast to kisspeptin, which is a key modulator of GnRH/LH pulse frequency (George et al. 2011), NK3R antagonist did not alter the frequency of LH pulses in this study, although the ability

to detect a change in LH pulse frequency may have been limited by the small number of men studied here, and the short 6 hour duration of frequent sampling. Consistent with some aspects of our findings, infusion of neurokinin B ligand in men elicited no effect on LH pulsatility (Jayasena et al. 2014c). On the other hand NK3R antagonist has been shown to reduce LH pulse frequency in women with PCOS (George et al. 2016) and in ovariectomised ewes (Fraser et al. 2015a, Li et al. 2015), although this was in state of fast LH pulsatility and in the animal studies NK3R antagonist was administrated centrally. Data on this in another state of high LH output- postmenopausal women- are presented in Chapter 4. Conversely, in the present study LH secretion was already low and there may be a threshold at which NK3R antagonist cannot suppress LH pulsatility further, accounting for the discrepancy observed, or a higher dose may be required. Moreover, inability to manipulate LH pulse frequency in men may reflect differences in male physiology in that changes in the frequency of LH pulses is not a prerequisite for normal testicular function, unlike its key role in regulating ovarian cyclicity (Filicori et al. 1986). Sexual dimorphism is well recognised in kisspeptin response, showing marked increase in LH with kisspeptin administration in men but variable response in women dependent on the sex-steroid milieu (Dhillo et al. 2007, George et al. 2012). Furthermore, the female hypothalamus has significantly more kisspeptin cell bodies and fibres compared to men (Hrabovszky et al. 2010), and there is a greater abundance of neurokinin B expressing neurones in ewes than rams (Goubillon et al. 2000), indicating gender differences in NKB-kisspeptin pathway. Lastly, loss of negative testosterone feedback is known to increase LH pulse frequency (Veldhuis et al. 2008), which may have balanced out the suppressive effect of NK3R antagonism on pulsatile LH secretion. Nevertheless, manipulation of pulsatile LH secretory pattern by the NK3R antagonist in this study, which is in concordance with stimulatory action of neurokinin B and inhibitory action of NK3R antagonist on LH pulses in animal studies (Fraser et al. 2015a, Li et al. 2015), supports the notion that neurokinin B acts centrally to modulate at least some aspects of pulsatile GnRH secretion and that there are complex neurokinin B/kisspeptin/GnRH interactions that result in the characteristics of the LH pulse.

KP-10 stimulated LH secretion in the presence of NK3R antagonist in normal men, which lends further support to the overall hierarchy whereby neurokinin B signalling is functionally upstream of kisspeptin in GnRH/LH pulse generation. This is consistent with the only human study to date investigating the hierarchical contribution of these neuropeptides in disease rather than health model, where KP-10 infusion restored pulsatile LH secretion in men and women with inactivating mutations in neurokinin B pathway (Young et al. 2013), and with equivalent results observed in animal studies (Ramaswamy et al. 2011, Garcia-Galiano et al. 2012). Although in the presence of NK3R antagonist, KP-10 stimulated LH concentrations were similar to those observed with KP-10 alone, there was some evidence that the KP-10 response was enhanced by the NK3R antagonist. This may reflect lower basal LH levels following pre-treatment with NK3R antagonist and perhaps a limit to the maximal KP-10 stimulatory effect. Conversely, co-infusion of neurokinin B itself and KP-54 in healthy men, stimulated gonadotropin and testosterone secretion significantly less than with KP-54 alone and had no effect on LH pulsatility (Narayanaswamy et al. 2016b). As downregulation in *GnRH* and *Kiss1r* mRNA was observed with senktide administration in rats (Grachev et al. 2012), it was proposed that in the presence of high levels of kisspeptin, neurokinin B acts to inhibit GnRH and kisspeptin transcription to reduce the stimulatory action of kisspeptin on gonadotropins (Narayanaswamy et al. 2016b). Together, this evidence suggests a more complex interaction between kisspeptin and neurokinin B in the regulation of GnRH secretion, raising the possibility of neurokinin B being a gatekeeper, which exerts both stimulatory and/or inhibitory downstream actions on kisspeptin in response to negative sex steroid feedback.

The present study has clear strengths in that a specific neurokinin-3 receptor antagonist was used, detailed LH pulse profiling was performed with blinded pulse analysis, and paired data was obtained for the control period and administration of NK3R antagonist. However, it is important to recognize that the sample size is small and a placebo control was not used. The limited suppression in the frequency of LH pulses by the NK3R antagonist might be due to the dose of AZD4901 being lower compared to doses used in animal studies. Direct comparison of human and animal

data requires caution since animal studies often use central administration of neuropeptides to delineate neurokinin B/kisspeptin/GnRH interaction. Peripherally administrated neuropeptides in this study may not have fully reached the hypothalamic regions of their action, although KNDy neurones send projections to the median eminence, which receives the portal circulation, and the arcuate nucleus, which have an incomplete blood-brain-barrier (Rodriguez et al. 2010).

In summary, using healthy men to explore the role of neurokinin B in a physiological state of slow LH secretory pattern, these data show that NK3R antagonism resulted in suppression of both LH and FSH concentrations, and a sustained decrease in testosterone secretion, indicating an important role for neurokinin B in the regulation of the male HPG axis. They show for the first time that a NK3R antagonist reduced some aspects of pulsatile LH secretion in men. Assessment of the interaction between neurokinin B and kisspeptin showed that LH response to kisspeptin was maintained in the presence of NK3R antagonist, supporting a hierarchical relationship of neurokinin B being predominantly proximal to kisspeptin. These data thus indicate that neurokinin B modulates GnRH/LH pulse generation in men but that there is a complex interaction between neurokinin B, kisspeptin and GnRH.

Chapter 4. Neurokinin B and Kisspeptin in the regulation of gonadotropin secretion in postmenopausal women

4.1 Introduction

In chapter 3, the interaction between kisspeptin and neurokinin B in modulation of pulsatile GnRH/LH secretion was investigated in a state of slow LH pulsatility. In healthy men, neurokinin B signalling was found to be predominantly upstream of kisspeptin, which is consistent with the stimulatory kisspeptin effect seen in patients with loss-of-function mutations in neurokinin B (*TAC3*) and neurokinin-3 receptor (*TACR3*) (Young et al. 2013) and equivocal findings from animal studies (Ramaswamy et al. 2011, Garcia-Galiano et al. 2012). However, there are no data on the role of NKB in states of high LH output in human health: postmenopausal women provide a model to investigate this.

Kisspeptin is a potent stimulator of LH secretion in men (Dhillo et al. 2005, George et al. 2011), the response in healthy women being variable and dependent on sex-steroid milieu across different phases of the menstrual cycle with a direct positive correlation with estrogen concentrations (Dhillo et al. 2007, George et al. 2012, Narayanaswamy et al. 2015). The only study administering kisspeptin in estrogen deficient postmenopausal women has shown a rather limited LH response to both KP-10 and GnRH injection compared to women in the follicular phase or those on hormonal contraception (George et al. 2012). This suggests that kisspeptin might already be operating at its maximal capacity to increase GnRH pulsatility and subsequently LH secretion in response to loss of negative ovarian feedback in postmenopausal women. In keeping with this, hypothalamic expression of *KISS1* and *TAC3* mRNA are upregulated, and kisspeptin and neurokinin B neurones are hypertrophied in the infundibular nucleus of postmenopausal compared to premenopausal women (Sheehan and Kovacs. 1966, Rance et al. 1990, Rance and Young. 1991, Rometo et al. 2007). Similarly, the hypothalamic expression of those neuropeptides is increased following an ovariectomy in rodents, ewes and monkeys but restored with estrogen replacement (Rance and Bruce. 1994, Abel et al. 1999, Sandoval-Guzman et al. 2004, Navarro et al. 2009, Eghlidi et al. 2010). Whether this

hypertrophy of kisspeptin and neurokinin B neurones and LH hypersecretion impact on the ability to manipulate the system is unknown.

In states of high LH pulsatility, such as in women with PCOS, and in animals models of ovariectomised ewes and castrate monkeys, selective blockade of NK3R with ESN364 or AZD4901 decreased the frequency of LH pulses, subsequently suppressing LH secretion and in women with PCOS lowering serum testosterone concentrations (Fraser et al. 2015a, Li et al. 2015, George et al. 2016). However, the inhibitory effect of such blockade on the NKB/KP/GnRH pathway and GnRH/LH pulsatility in high LH secretion in healthy women, such as post menopause, has not yet been explored.

Withdrawal of estrogen and a compensatory increase in LH secretion in menopause is also accompanied by hot flushes in some women, the mechanisms of which remain unclear. Hot flushes are characterised by sudden onset of transient and intense sensation of heat associated with heat-loss response, including cutaneous vasodilation and sweating. A hypothalamic mechanism for this was first proposed almost 20 years ago, when using an ingestible thermometer small elevations in body core temperature were detected preceding most (65-76%) hot flushes (Freedman. 1998). Hot flushes triggered by heat and exercise induced small increases in core body temperature, which in symptomatic postmenopausal women were acting within a lowered thermoregulatory threshold (0°C) for heat dissipation than in postmenopausal women who do not flush (thermoregulatory threshold (0.4°C) (Freedman. 1998), although these data have not been replicated.

Studies involving blood sampling at frequent intervals have revealed the synchronisation of LH pulses with the onset of hot flushes in women (Casper et al. 1979, Oakley et al. 2015). It is well established that pulsatile LH secretion is a marker of GnRH pulsatility, and functional links between pathways driving GnRH pulsatility and vasomotor symptoms have been proposed (Rance et al. 2013). KNDy neurones have been implicated as an important part of the GnRH pulse generator, they are sensitive to estrogen withdrawal and project to the thermoregulatory region within the median preoptic area (Krajewski et al. 2010), suggesting the role of KNDy signalling in the aetiology of flushes. Manipulation of high LH pulsatility in

postmenopausal women by a NK3R antagonist may therefore open new paradigms in the management of menopausal hot flushes. Consistent with the above is the demonstration that the administration of exogenous neurokinin B induced hot flushes in healthy premenopausal women (Jayasena et al. 2015b). Ablation of NKB-expressing neurones reduced cutaneous vasodilation in rodents (Mittelman-Smith et al. 2012), and NK3R antagonist lowered body core temperature in sheep (Fraser et al. 2015a), whilst NK3R agonist senktide administered directly into the rat median preoptic area was associated with increased tail vasodilatation (Dacks et al. 2011). Collectively, these findings suggest the involvement of NKB signalling in vasomotor symptoms as well as GnRH/LH pulse generation, having potential for novel therapeutic application in the treatment of hot flushes. Using a selective NK3R antagonist, the role of neurokinin B in the regulation of hypergonadotropic state in menopausal women and its interaction with kisspeptin was therefore investigated.

4.2 Objectives

The objectives of this study were in healthy postmenopausal women:

1) To determine the effect of NK3R antagonist on LH and FSH secretion;
2) To investigate the effect of NK3R antagonist on LH pulsatility;
3) To investigate the effect of KP-10 on gonadotropin secretion and whether this response is preserved in the presence of NK3R antagonist, thereby revealing the functional hierarchy of neurokinin B being proximal to kisspeptin;
4) To record changes in hot flushes in susceptible women during administration of NK3R antagonist.

4.3 Methods

4.3.1 Participants

Eleven healthy postmenopausal women, aged 46-62 years and 2-20 years since natural menopause were recruited to this study; all volunteers provided informed

written consent. Subjects were on no hormonal replacement therapy and not taking any preparations for hot flushing. As the primary outcome was analysis of LH secretion, the presence or frequency of hot flushes were not inclusion criteria, and was reported in 8 of the subjects. Subjects were eligible based on inclusion and exclusion criteria (Table 2.1 Inclusion and exclusion criteria, Chapter 2), had normal physical examination, and full blood count, renal function, electrolytes, liver function and electrocardiogram were within normal limits.

4.3.2 Study drugs

The specific NK3R inhibitor AZD4901 was administered orally at 40 mg twice daily. Kisspeptin-10 was administered as an intravenous bolus at 0.3 μg/kg and prepared as described in Chapter 2 (2.3.1 Kisspeptin-10).

4.3.3 Protocol

Investigation of the effect of NK3R antagonism on LH and FSH secretion.

Schematic presentation of the protocol is shown in Figure 4.1. Study design is similar to that used in healthy men (Chapter 3). Volunteers were administered the NK3R antagonist for 7 days. Peripheral venous blood was sampled for spot LH and FSH 24 hours before treatment (pre-treatment) and on days 2, 4, 6 and 7 of NK3R antagonist administration. Once daily blood sampling was performed in the morning: during treatment, this was immediately prior to the next dose of NK3R antagonist, i.e. 12 hours after the previous dose.

Investigation of the effect of neurokinin B on LH pulsatility.

On the pre-treatment day and on the last day of NK3R antagonist administration (day 7) volunteers attended our clinical research facility for 8 hours. All visits commenced between 0800 and 0900 hours to avoid diurnal variation. Blood samples were collected via an indwelling intravenous cannula at 10 min intervals for 6 hours for the assessment of LH pulsatility. The dose of NK3R antagonist was administered immediately prior to the start of sample collection (day 7).

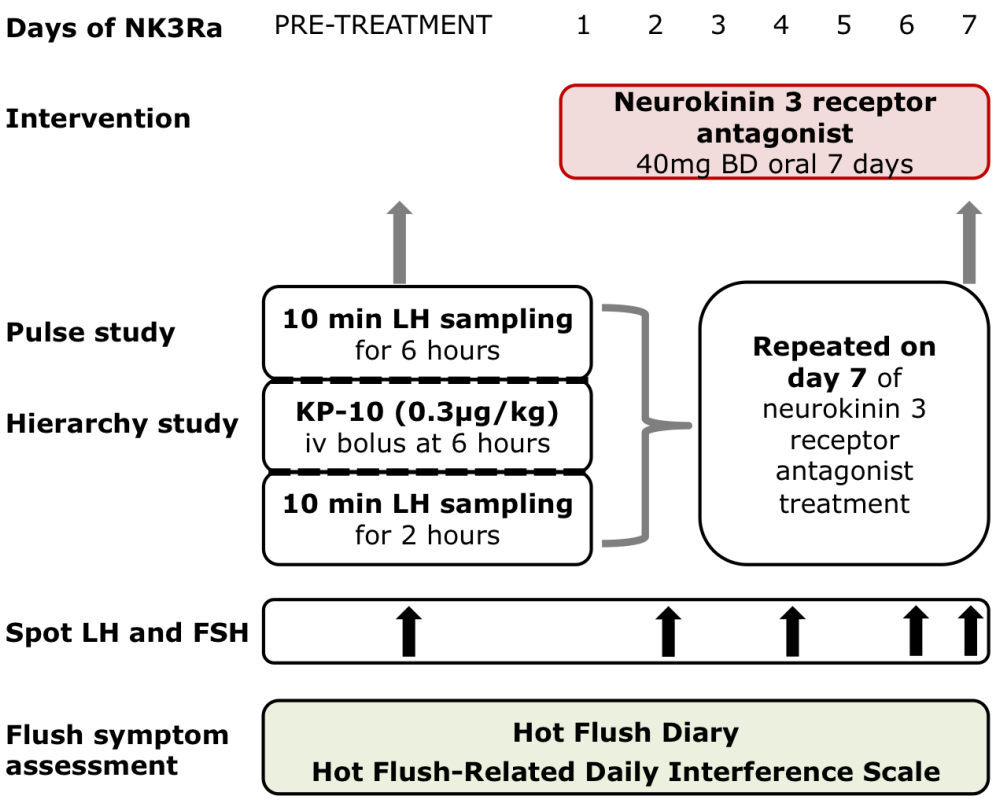


Figure 4.1 Study protocol.

11 healthy postmenopausal women were administered NK3R antagonist AZD4901 orally for 7 days. LH and FSH were measured throughout the study. LH pulsatility was assessed during 10 minute blood sampling for 6 hours on the day before and on the last day of NK3R antagonist treatment. KP-10 was administered as an intravenous bolus at 6 hours with further frequent blood sampling for 2 hours. Women self-reported the frequency and severity of hot flushes and the interference of those flushes with daily activities throughout the study.

Establishment of the hierarchy of kisspeptin and neurokinin B on gonadotropin secretion in postmenopausal women.

Kisspeptin-10 0.3 µg/kg intravenous bolus was administered after 6 hours of the pulsatility study as above with further blood sampling for 2 hours every 10 min for LH and hourly for FSH.

The role of neurokinin B in the modulation of flush symptoms.

This formed the secondary outcome of the study. Subjects were not specifically informed that the study drug may reduce the experience of hot flushes. Subjects continued their usual lifestyle whilst taking part in the study, including intake of hot

drinks, caffeine, alcohol and spicy food. Subjects were asked to self-report any flushing symptoms (e.g. heat and sweating) and night-time awakenings, including their frequency and perceived severity (1 ‘mild’= heat sensation and no sweating; 2 ‘moderate’= heat and sweating, not causing disruption to activity; 3 ‘severe’ = heat and sweating, disrupting activity) by using ‘Hot Flush’ diaries for a week prior to study visits and whilst on NK3R antagonist treatment (Fig. 4.2). Subjects were encouraged to keep the diary with them at all times and record the flush and its severity after it occurred to minimise ‘retrospective monitoring’. Night-time awakenings were recorded in the morning. The subjective hot flush-related daily interference scale (HFRDIS) (Carpenter. 2001), covering the preceding 7 days, was completed by the subjects on the last day of pre-treatment period and upon the completion of NK3R antagonist (Fig. 4.2). HFRDIS is a 10-item scale allowing subjective assessment of the perceived affect of flushes on a number of activities of daily living and the quality of life (Carpenter. 2001). Subjects were asked to rate the bothersomeness of their hot flushes on each item of activity using the scale from 0 (do not interfere) to 10 (completely interfere).

Safety profile.

Safety blood tests including full blood count, renal function and electrolytes, and liver function were checked before commencing the NK3R antagonist, at the end of each 8 hour visit for frequent blood sampling and 2-3 weeks after the treatment had finished.

Hot Flush Diary

Study Number:

Week beginning:

Please complete this information for each day of the week
Mild flush = heat, no sweating
Moderate flush = heat and sweating, not causing disruption to activity
Severe = heat and sweating, disrupting activity

Day and Date	No of flushes	Severity of flushes 1=mild 2=moderate 3=severe	No. of night time awakenings

Please circle one number to the right of each phrase to describe how much DURING THE PAST WEEK hot flushes have INTERFERED with each aspect of your life. Higher numbers indicate more interference with your life.

If you are not experiencing hot flushes or if they do not interfere with these aspects of your life, please mark the "0" to the right of each question.

Hot Flush Related Daily Interference Scale

	Do Not Interfere 0 1 2 3 4 5 6 7 8 9 10 Completely Interfere
1. Work(outside the home and housework)	
2. Social activities (time spent with family/friends etc)	
3. Leisure activities (time spent relaxing, hobbies etc.)	
4. Sleep	
5. Mood	
6. Concentration	
7. Relations with others	
8. Sexuality	
9. Enjoyment of life	
10. Overall quality of life	

Figure 4.2 'Hot Flush' diary and Hot Flush-Related Daily Interference Scale questionnaire.

4.3.4 Analytical methods

Hormone assays, pulsatile LH secretion and safety blood tests were performed as described in Chapter 2. The frequency of hot flushes was calculated as the mean number of hot flushes recorded in a 24-hour period and the mean number of night-time awakenings as a result of those flushes (Freeman et al. 2011). Severity of hot flushes was calculated by selecting the highest severity rating for hot flushes for each subject in a 24-hour period and averaging the score for the week (Freeman et al. 2011). For HFRDIS mean total score as well as individual item score were calculated (Carpenter. 2001).

4.3.5 Statistical analysis

Data were tested for normality by Shapiro-Wilk normality test. Mean LH and FSH concentrations over time were compared using one-way ANOVA followed by Bonferroni's multiple comparisons post hoc analysis. Area under the curve (AUC) LH and FSH during 8 hours of frequent LH sampling (every 10 minutes) and FSH sampling (every hour) was determined by trapezoid integration on the pre-treatment day (control) and on day 7 of NK3R antagonist administration. Comparisons in AUC across time and between the groups were performed using repeated-measures two-way ANOVA with Bonferroni's multiple comparison post hoc analysis. AUC hourly gonadotropin response to KP-10 was not calculated for one of the eleven women, as full 2-hour sampling data post KP-10 injection was not obtained due to failure of indwelling intravenous cannulas and inability to secure further intravenous access.

Parameters of LH pulsatility were compared by paired Student's t-test (for normally distributed data: secretory mass per pulse, basal and pulsatile secretion, ApEn) or Wilcoxon matched-pairs signed rank test (for data that did not have a normal distribution: pulse frequency).

Paired mean frequency of hot flushes and night-time awakenings were compared using Wilcoxon matched-pairs signed rank test. Mean flush severity scores (unpaired as score was set to missing on the day that hot flushes equalled zero) were compared using Mann-Whitney test. Total and individual item HFRDIS scores were compared by Wilcoxon matched-pairs signed rank test. Data are presented as mean ± SEM.

4.4 Results

4.4.1 NK3R antagonist decreases LH but not FSH secretion

Analysis of single time point blood samples (with sampling before drug administration) showed that treatment with NK3R antagonist had a marginal suppressive effect on LH secretion (ANOVA $p=0.008$), lowering it on day 7 of treatment (pre-treatment 29.5±4.1 vs 24.4±3.8 IU/l day 7, $p<0.05$) (Fig. 4.3A) with no difference from pre-treatment on other sampling days. A more detailed analysis of LH secretion every 10 minutes for 6 hours pre-treatment and on day 7 of NK3R antagonist administration showed no difference in AUC LH (Fig. 4.3B). FSH secretion was unchanged by the NK3R antagonist (Fig. 4.3C).

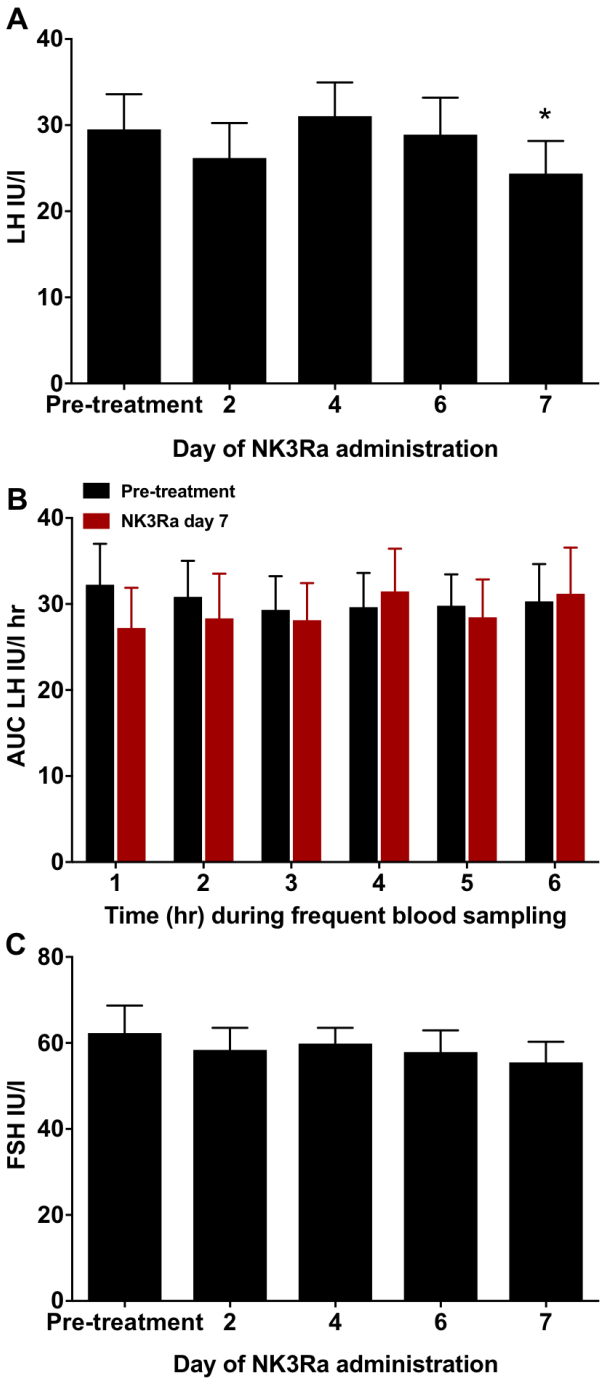


Figure 4.3 Gonadotropin response to administration of NK3R antagonist in healthy postmenopausal women.

(A) LH concentrations during 7 day course of NK3R antagonist treatment (blood samples taken once daily) and **(B)** AUC LH during 6 hours of 10 minute LH sampling pre-treatment and on day 7 of NK3R antagonist administration. **(C)** FSH response to NK3R antagonist. Data are presented as mean ± SEM. * $p<0.05$ vs pre-treatment.

4.4.2 NK3R antagonist modulates pulsatile LH secretion in postmenopausal women

An example of an LH pulse frequency profile is shown in figure 4.4 (A). Consistent with reduced mean LH secretion on day 7 of treatment, deconvolutional analysis showed that the NK3R antagonist decreased basal (i.e. nonpulsatile) LH secretion (p=0.006), although LH pulse frequency did not change with NK3R antagonist in the group as a whole (0.8±0.1 vs 0.7±0.1 pulses/hr, ns) (Fig. 4.4 B and C). Secretory mass per LH pulse was increased with NKR3 antagonist treatment (p=0.01) (Fig. 4.4D) with no overall effect on total amount of LH secreted in a pulsatile manner (Fig. 4.4E) and there was no change in ApEn, the orderliness of LH secretory pattern (Fig. 4.4F).

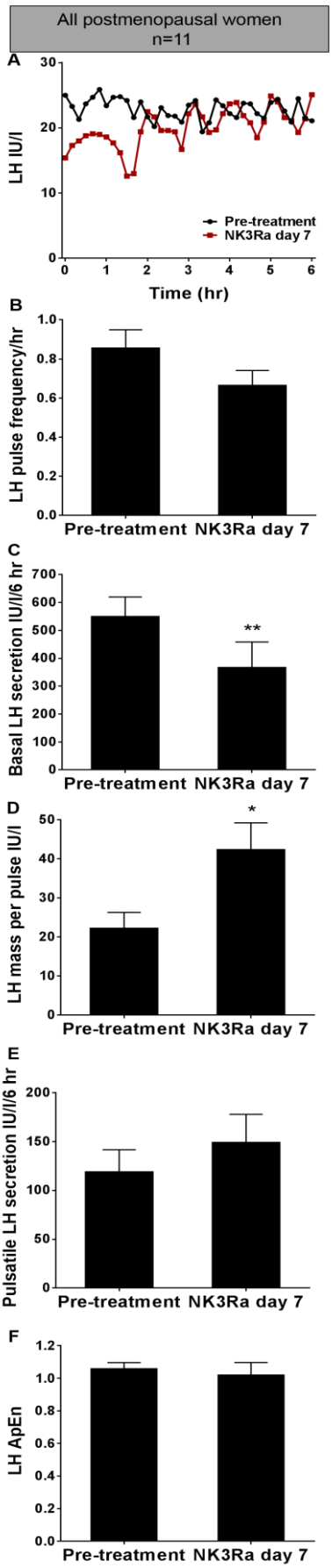


Figure 4.4 Analysis of 6 hour LH secretory pattern on day 7 of NK3R antagonist treatment compared to control pre-treatment day in healthy postmenopausal women.

Illustrative LH pulse profile from one subject **(A)** undergoing 10 minute blood sampling for LH for 6 hour with no NK3R antagonist treatment (*black circles*) and on day 7 of NK3R antagonist treatment (*red squares*). LH pulse frequency **(B)**, basal (nonpulsatile) LH secretion **(C)**, mass of LH per pulse **(D)**, pulsatile LH secretion **(E)** and the relative orderliness/regularity of LH secretory pattern **(F)** on day 7 of NK3R antagonist treatment was compared to pre-treatment day in all postmenopausal women (n=11). Data are presented as mean ± SEM. *, p<0.05; **, p<0.01.

4.4.3 KP-10 does not elicit gonadotropin response with or without NK3R antagonist

Intravenous KP-10 was administered at 6 hours of frequent sampling, with 2 hours sampling thereafter. KP-10 had no effect on LH and AUC LH and FSH secretion in postmenopausal women, and this response was not affected by the NK3R antagonist treatment (Fig. 4.5).

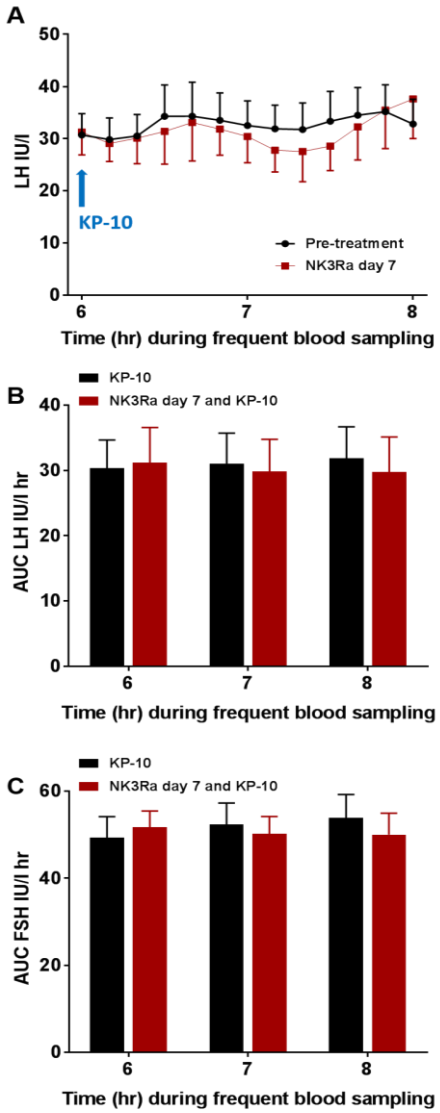


Figure 4.5 LH, AUC LH and AUC FSH response to KP-10 injection with and without NK3R antagonist in postmenopausal women.

Mean LH **(A)** and AUC LH (sampling every 10 minutes) **(B)** and AUC FSH (sampling every hour) **(C)** were compared over one hour before KP-10 administration (i.e. at 6 hours) with 2 hours post KP-10 administration, before and after 7 days of NK3R antagonist treatment during frequent blood sampling (n=10). Data are presented as mean ± SEM.

4.4.4 NK3R antagonist reduces self-reported postmenopausal hot flushes

Hot flush frequency, severity and interference with daily activities were recorded in the 8 of 11 postmenopausal women experiencing these symptoms, for 7 days pre-treatment and whilst on NK3R antagonist. These women reported a reduction in hot flush frequency (3.4±1.2 to 1.0±0.6 hot flushes/day with NK3Ra, p=0.008) and night-time awakenings as a result of those flushes (1.6±0.3 to 0.4±0.2 awakenings/night with NK3Ra, p=0.008) whilst on NK3R antagonist (Fig. 4.6 A and B). There was also an improvement in the severity of flushing symptoms from moderate (heat and sweating, not causing disruption to activity) to mild (heat and no sweating) (mean severity 2.1±0.2/3 vs 1.4±0.1/3 with NK3Ra, p=0.03) with the NK3R antagonist (Fig. 4.6C). The time-course of changes in day-time hot flushes across 7 days of NK3R antagonist administration was analysed ($p<0.0001$), showing significantly lower hot flush frequency on the 2nd day of treatment ($p<0.05$ vs baseline) and thereafter each day for the remainder of NK3Ra administration ($p<0.05$ at day 3-7 vs baseline) (Fig. 4.6D). Night-time awakenings as a result of those flushes also showed a rapid decrease in frequency with the NK3R antagonist and were reduced throughout treatment (p=0.0003) (Fig. 4.6E).

NK3R antagonist reduced total hot flush-related daily interference scale (HFRDIS) score (31.3±7.7 vs 9.0±4.7, p=0.008), indicating significantly reduced interference of hot flushes with daily function (Table 4.1). The highest mean interference rating was noted for sleep, which was improved by the NK3R antagonist (6.4±0.8 vs 1.1±0.6, p=0.008). Whilst all other individual HFRDIS items also showed a reduction in the interference scores, they did not reach statistical significance.

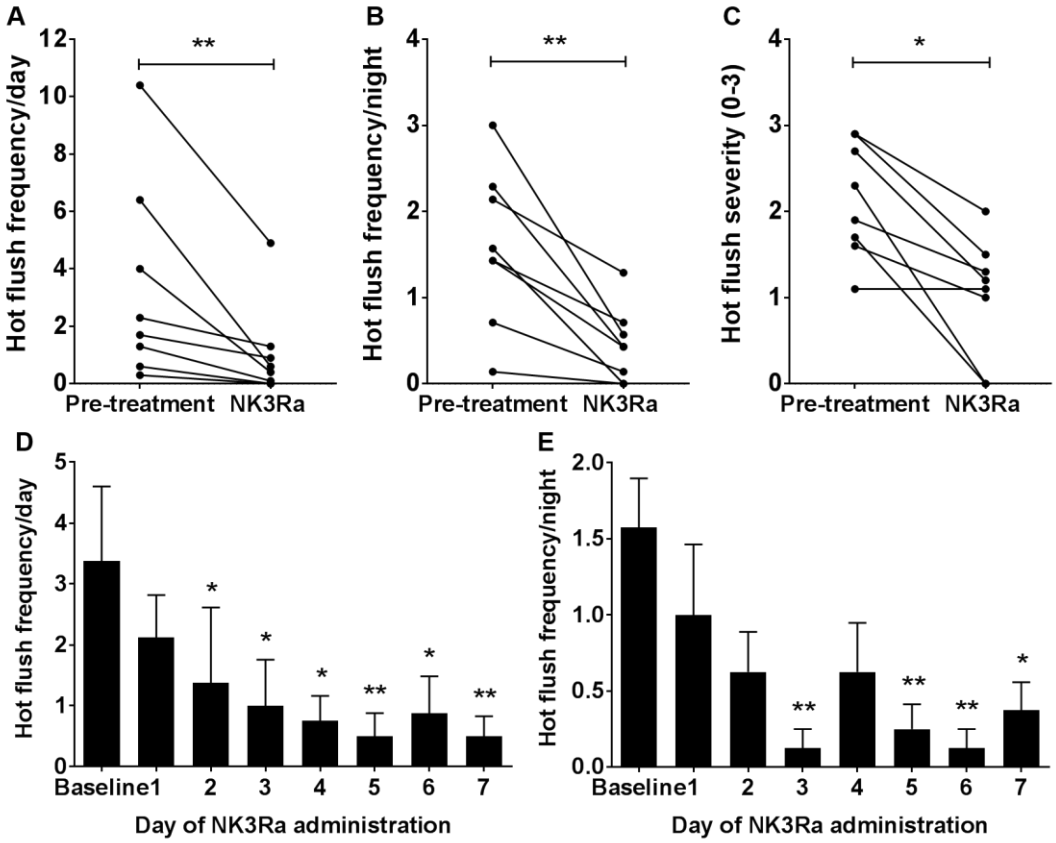


Figure 4.6 Differences in the frequency and severity score of menopausal hot flushes pre-treatment and during 7 days of NK3R antagonist administration.

Response to NK3R antagonist is shown for individual postmenopausal women for the frequency of day **(A)** and night-time **(B)** hot flush and their severity **(C**) over 7 days before and during NK3R antagonist administration. Time-course in the reduction of day **(D)** and night-time **(E)** hot flush frequency over 7 days of NK3R antagonist administration is compared to pre-treatment period. Data are presented as mean ± SEM.*, $p<0.05$; **, $p<0.01$.

Hot Flush Interference	Pre-treatment (n=8)	NK3Ra (n=8)	p value
Total Hot Flush-Related Daily Interference Scale (HFRDIS) Score	31.3 (7.7)	9.0 (4.7)	0.008
Individual HFRDIS item responses (0-10; do not interfere-completely interfere)			
Work	5.8 (1.1)	1.8 (1.1)	0.25
Social activities	5.5 (1.0)	1.5 (1.2)	0.13
Leisure activities	4.8 (0.9)	1.5 (1.2)	0.14
Sleep	6.4 (0.8)	1.1 (0.6)	0.008
Mood	4.8 (0.8)	1.4 (0.7)	0.06
Concentration	5.8 (0.8)	1.6 (0.7)	0.06
Relations with others	2.8 (1.1)	0.3 (0.3)	0.25
Sexuality	4.8 (1.0)	2.6 (1.2)	0.25
Enjoyment of life	3.7 (0.8)	1.2 (0.6)	0.06
Overall quality of life	4.2 (0.7)	1.3 (0.6)	0.06

Table 4.1 Differences in hot flush-related daily interference scale scores pre-treatment and during 7 days of NK3R antagonist administration.

Total and individual item HFRDIS scores in 8 postmenopausal women are shown. Data are presented as mean (SEM).

4.4.5 NK3R antagonist decreases LH pulse frequency in postmenopausal women with hot flushes

Figure 4.7 (A and G) shows examples of LH pulse frequency profile of a postmenopausal woman reporting hot flushes and one not having such symptoms. Although LH pulse frequency was not affected by the NK3R antagonist in a group as a whole (Fig. 4.4), in a secondary analysis of the 8 of 11 women with self-reported symptomatic hot flushes, NK3R antagonist reduced both basal LH secretion ($p=0.03$) and the frequency of LH pulses (1.0±0.1 vs 0.7±0.1 pulses/hr, $p<0.05$) (Fig. 4.7 H and I). The mass of LH per pulse was increased ($p=0.04$), although pulsatile LH secretion remained unchanged (Fig. 4.7 J and K). The orderliness (ApEn) of LH secretory pattern was unaffected by treatment in those with flush symptoms (Fig. 4.7L).

4.4.6 Tolerability and safety

NK3R antagonist was well tolerated with no subject discontinuing the treatment during the study, and no adverse effects were reported during KP-10 administration. Haematology and biochemistry safety parameters remained stable in all subjects throughout the study period.

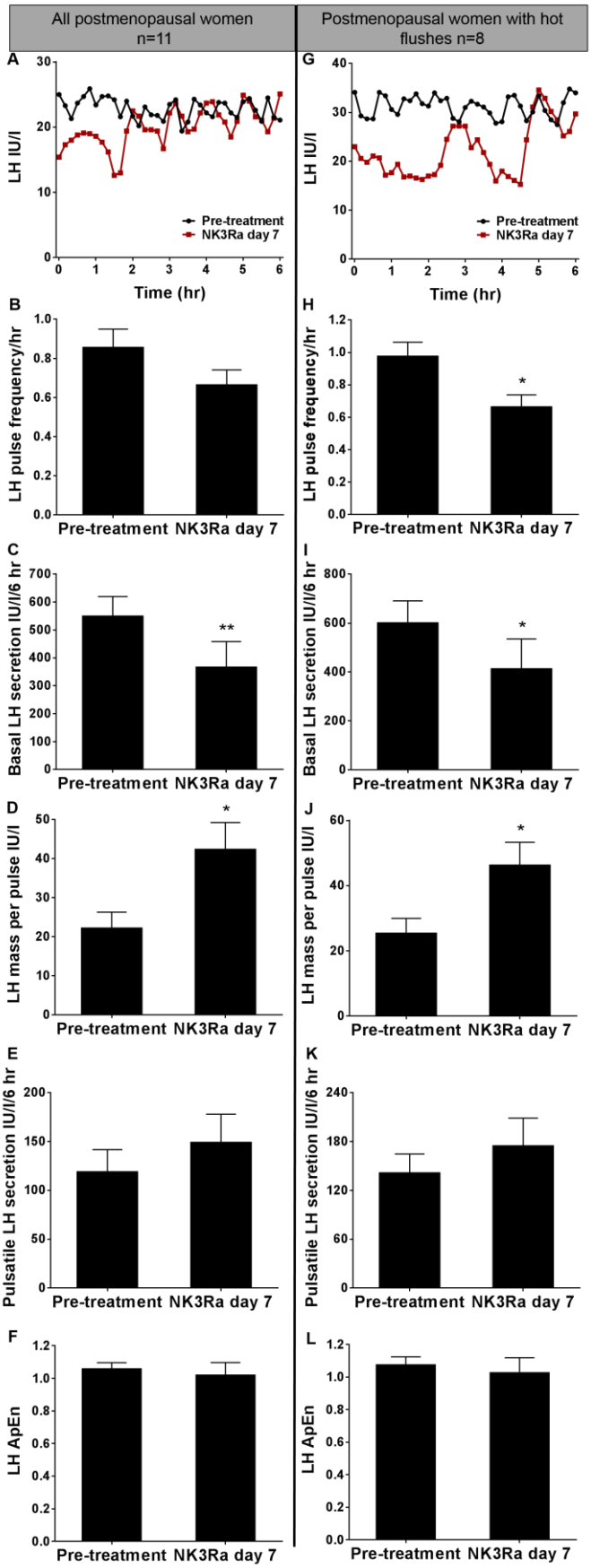

All postmenopausal women n=11
Postmenopausal women with hot flushes n=8
A
G
LH IU/l
Pre-treatment
NK3Ra day 7
Time (hr)
B
H
LH pulse frequency/hr
C
I
Basal LH secretion IU/l/6 hr
D
J
LH mass per pulse IU/l
E
K
Pulsatile LH secretion IU/l/6 hr
F
L
LH ApEn
Pre-treatment NK3Ra day 7

Figure 4.7 Comparison of LH secretory pattern with and without NK3R antagonist treatment in healthy postmenopausal women as a group and in those experiencing hot flushes only.

Illustrative LH pulse profile from two subjects **(A with no flushes, G with flushes)** undergoing 10 minute blood sampling for LH for 6 hour with no NK3R antagonist treatment (*black circles*) and on day 7 of NK3R antagonist treatment (*red squares*). LH pulse frequency **(B and H)**, mean basal (nonpulsatile) LH secretion **(C and I)**, mass of LH per pulse **(D and J)**, pulsatile LH secretion **(E and K)** and the relative orderliness/regularity of LH secretory pattern **(F and L)** on day 7 of NK3R antagonist treatment was compared to pre-treatment day in all postmenopausal women (n=11) and in a subgroup of women reporting hot flushes (n=8). Note that data in left panel is taken from Fig. 4.4 for the ease of pulsatile LH secretion comparison between all women versus those with subjective hot flushes. Mean ± SEM**.** *, $p<0.05$; **, $p<0.01$.

4.5 Discussion

This study has investigated for the first time the role of neurokinin B and its interaction with kisspeptin in the regulation of GnRH and LH secretion in a state of high LH pulsatility in health, the postmenopausal woman. Selective blockage of NKB signalling had only a very limited suppressive effect on LH secretion as assessed on daily sampling with no detected effect on FSH release. Blood sampling during NK3R antagonist administration was performed before the morning dose, i.e. 12 hours after the previous treatment and may have missed subtle changes in gonadotropin secretion. The importance of the sampling schedule is indicated by the significant effect of the NK3R antagonist on parameters of pulsatile LH secretion, which were measured over 6 hours immediately following drug administration. The NK3R antagonist reduced basal LH secretion, demonstrating the involvement of NKB signalling in the regulation of the hypergonadotropic state in postmenopausal women. The effect of NKB on pulsatile GnRH secretion is further supported in a subgroup of women experiencing hot flushes where a reduction in LH pulse frequency was identified. These women also reported a marked reduction in flush symptoms whilst on NK3R antagonist consistent with a link between GnRH/LH pulsatility and vasomotor symptoms (Rance et al. 2013).

These data show that in postmenopausal women NK3R antagonist preferentially decreased LH but not FSH secretion. This pattern of effect of NK3R antagonist is consistent with data from patients with inactivating mutations in NKB pathway who display reduced LH levels but near normal FSH secretion, likely mediated through decreased GnRH pulse frequency (Young et al. 2013). While the present study has shown that in postmenopausal women high LH output can be altered by the NK3R antagonist, LH pulse frequency in the group as a whole, the proportion of total LH secreted in pulses and the regularity of LH secretion were not affected. Mass of LH secreted per pulse was increased with the NK3R antagonist, although this may be due to LH pulses merging in postmenopausal women without return to basal secretion thereby giving an apparent greater LH secretory mass per pulse. However, the LH response in this study is in contrast to the only previous data from a high LH output state in humans, which is in women with PCOS, where the frequency of LH

pulses was decreased by the NK3R antagonist after 7 days administration at the dose used here (George et al. 2016) and similar findings in ovariectomised ewes using a different NK3R antagonist MRK-08 (Li et al. 2015). Duration and/or dose of NK3R antagonist administration in this study may have contributed to the limited suppressive effect seen on LH/GnRH secretion in contrast to hypogonadotropic hypogonadism observed in patients with loss-of-function mutations in NKB and its receptor (Young et al. 2013) or NK3R antagonist administration for 4 weeks in women with PCOS (George et al. 2016). Additionally, pulsatility analysis in high frequency states can be challenging, with discrimination of discrete pulses difficult. Inability to manipulate high LH secretion by the NK3R antagonist in menopausal women may reflect estrogen depletion, and high activity of the NKB pathway. Increased expression of hypothalamic *TAC3* mRNA after ovariectomy in animal models is restored with estrogen replacement (Rance and Bruce. 1994, Abel et al. 1999, Sandoval-Guzman et al. 2004, Navarro et al. 2009, Eghlidi et al. 2010), and sex-steroid receptors are known to localise to the NKB perikarya (Rance et al. 1990, Rance and Young. 1991), clearly supporting a role of estrogenic feedback in the regulation of NKB signalling. It seems plausible that loss of negative estrogen feedback following the menopause impedes the ability to manipulate the NKB/KP/GnRH pathway as readily as in other states. Although LH secretion was reduced with the NK3R antagonist in an animal model of menopause, the ovariectomised ewes (Li et al. 2015) and in castrate monkeys (Fraser et al. 2015a), treatment was administrated centrally, and species difference precludes direct comparison of the result, especially since there is anatomical variation in pathways mediating sex-steroid feedback between the humans and other mammals (Smith et al. 2005a, Smith et al. 2006b, Rometo et al. 2007, Herbison. 2008).

The lack of KP-10 effect on gonadotropin secretion seen in this study is consistent with previous data showing minimal LH response to both KP-10 and GnRH injection in postmenopausal women as opposed to women in the early follicular phase of the menstrual cycle or taking hormonal contraceptives (George et al. 2012). This further highlights the role of sex-steroid feedback in the control of hypothalamic neuropeptide signalling. It seems likely that due to loss of negative estrogen

feedback post menopause, the kisspeptin system is already operating at its maximum to increase GnRH and LH secretion with little scope for further a stimulatory effect of exogenous kisspeptin. This is supported by hybridization histochemistry showing that in the postmenopausal infundibular nucleus kisspeptin neurones are hypertrophied, their number is increased, and they have increased expression of both *KISS1* and *TAC3* mRNA (Sheehan and Kovacs. 1966, Rance et al. 1990, Rance and Young. 1991, Rometo et al. 2007). The absence of an effect of KP-10 on LH alone or in the presence of NK3R antagonism precludes clear analysis of the hierarchical relationship between kisspeptin and NKB in the postmenopausal women. It would be of interest to repeat the experiment in postmenopausal women taking estrogen replacement.

Interestingly, the NK3R antagonist reduced LH pulse frequency in postmenopausal women reporting hot flushes only, despite the smaller sample group. It is however unclear as to whether NKB acts to modulate GnRH/LH pulsatility specifically in those experiencing flush symptoms, as larger groups would be required to explore this more robustly. In this study, postmenopausal women were not recruited based on their hot flushes and statistical comparisons between those with and without flushing were not feasible since there were only 3 women in the latter subgroup. Small sample size may have impacted overall effect seen on LH pulsatility. It is however possible that the NKB pathway is somewhat different and is enhanced in women with hot flushes, thereby being more responsive to suppression by the NK3R antagonist.

The finding that all postmenopausal women with symptomatic flushes (8/11) reported a reduction in the frequency of total and night-time hot flushes whist on NK3R antagonist and an improvement in their severity from moderate to mild is a striking and novel finding. The response to NK3R antagonist in the reduction of flush symptoms was also a rapid one, with a significant fall in both day time and night time symptoms after only 2 days of treatment. The NK3R antagonist also reduced the interference of those flushes with daily activities. The present data implicate NKB as a key link between sex-steroid deficiency and hot flushes, as proposed from studies in animal models (Rance et al. 2013). In premenopausal

women neurokinin B itself administered as an intravenous infusion over 30 minutes induced the sensation of heat, which was accompanied by increased heart rate and skin conductance, resembling events associated with menopausal hot flushes (Jayasena et al. 2015b). Moreover, a recent genome-wide association study has localised single-nucleotide polymorphisms associated with vasomotor symptoms to the neurokinin 3 receptor locus (*TACR3*) (Crandall et al. 2016). Reduced cutaneous vasodilation in rodents with ablation of NKB-expressing neurones (Mittelman-Smith et al. 2012) and lowered body core temperature in NK3R antagonist treated sheep (Fraser et al. 2015a) lend further support for the involvement of NKB signalling in hot flushes. Reduction in the frequency of LH pulses by the NK3R antagonist observed here suggests the mechanism of flushes to be tied to the hypothalamic control of pulsatile GnRH secretion, although the degree of fall in LH pulsatility was much less than the fall in hot flush frequency. This is consistent with studies showing the synchronization of LH pulses with hot flushes in women (Casper et al. 1979, Rance et al. 2013, Oakley et al. 2015). Although these data support the involvement of NKB in vasomotor symptoms, it remains possible that separate pathways exist mediating hot flushes and those associated with pulsatile GnRH/LH secretion, with both involving NKB regulation.

The limitation of the absence of a placebo group in the present study is acknowledged, as well as the small number of women studied. Placebo has been shown to reduce hot flushes by 20-30% within 4 weeks of treatment and in 15% of women experiencing symptomatic flushes (Sloan et al. 2001, Boekhout et al. 2006). In contrast, NK3R antagonist treatment in this study was associated with approximately 70% reduction in hot flushes over only 7 days of treatment and in all 8 women reporting hot flushes. Subjective reporting of hot flushes is currently the most accurate method for detection and assessment of those flushes and is the method of choice in clinical studies in the field (Guttuso et al. 2012, Sievert. 2013, Joffe et al. 2014). Objective assessment of menopausal hot flushes, such as using skin conductance monitors would have reinforced the findings but was not performed in this study. This group had a lower hot flush frequency than is generally the case in studies specifically investigating therapies for that condition (Carpenter.

2001), reflecting recruitment criteria not specifying the presence of or a minimum flush frequency. Whether NK3R antagonist has a therapeutic potential in the non-steroidal management of hot flushes remains to be established. Nevertheless, the findings above form a strong basis for this.

In summary, a state of high LH secretory pattern in postmenopausal women is relatively refractory to manipulation by the NK3R antagonist and kisspeptin but effects on LH pulsatility were demonstrated. The absence of a response to kisspeptin-10 precluded investigation of the relationship between the NKB and kisspeptin pathways in the postmenopause. We have shown that NK3R antagonist reduced subject reported menopausal hot flushes and reduced the frequency of LH pulses in those women. These data demonstrate that NKB signalling is involved in the regulation of LH secretion in the hypergonadotropic state of the menopause and provide indirect evidence linking vasomotor symptoms and high GnRH/LH pulse frequency to the NKB pathway. Although our data are based on small numbers and have limitations, NK3R antagonism may have clinical application in management of hot flushes.

Chapter 5. Neurokinin B in the regulation of ovarian function in healthy women in the early follicular phase of menstrual cycle

5.1 Introduction

In Chapter 3 and 4, the role of NKB in states of slow and high LH pulsatility was investigated. In healthy men and postmenopausal women NKB action was found to be through GnRH secretion by modulating its basal i.e. nonpulsatile release with no effect of the frequency of LH/GnRH pulses in those groups. Whilst changes in pulsatile LH/GnRH secretion had a marked effect on serum LH and testosterone levels in men, manipulation of the NKB/GnRH/gonadotropin pathway was rather refractory in postmenopausal women. This suggests that sex-steroid feedback is important in modulating downstream effects of NKB on GnRH secretion, although sex-specific differences in the importance of the NKB pathway may also be relevant, given the differences in the number of NKB expressing neurones (Goubillon et al. 2000), their fibre distribution (Ciofi et al. 2006) and postnatal development of hypothalamic NKB neuronal network (Ciofi et al. 2007) in males compared to females. However, the involvement of NKB in GnRH/LH pulsatility with the varying sex-steroid milieu across the menstrual cycle in healthy premenopausal women has not yet been explored.

NKB is a key modulator of GnRH and hence gonadotropin secretion, as loss-of-function mutations in genes encoding neurokinin B (*TAC3*) and neurokinin-3 receptor (*TACR3*) result in hypogonadotropic pubertal delay (Topaloglu et al. 2009). However, the converse stimulatory effect of NKB on gonadotropin secretion was not seen in the only human study to date administering NKB as an intravenous infusion over 3 hours during the follicular, pre-ovulatory and mid-luteal phases of the menstrual cycle in healthy women (Jayasena et al. 2014c). Data from animal studies are also inconclusive with both stimulatory and inhibitory effects of NKB reported (Billings et al. 2010, Navarro et al. 2011a, Ramaswamy et al. 2011). In rodents, neurokinin B decreased LH secretion in ovariectomised animals (Sandoval-Guzman and Rance. 2004, Navarro et al. 2009), but in intact male mice had no effect on LH

secretion (Corander et al. 2010), whereas in diestrus and proestrus female rats NK3R agonist induced LH secretion (Navarro et al. 2011a), suggesting the action of neurokinin B to be dependent on circulating sex-steroids. In higher species, the stimulatory role of neurokinin B on LH secretion is more consistent and has been observed in ewes (Billings et al. 2010, Li et al. 2015), goats (Wakabayashi et al. 2010) and monkeys (Ramaswamy et al. 2010).

Similarly to patients with inactivating mutations in the NKB pathway (Topaloglu et al. 2009), studies involving selective blockade of NK3R have been more consistent in demonstrating a suppressive effect on LH but not FSH secretion. When administrated to gonadectomised ewes, the NK3R antagonist (ESN364 and MRK-08) decreased LH secretion and pulse frequency (Fraser et al. 2015a, Li et al. 2015) with similar findings observed in women with PCOS administered AZD4901, a condition also characterised by high LH pulse frequency (George et al. 2016). Administration of the NK3R antagonist ESN364 throughout the follicular phase in intact female monkeys inhibited estradiol secretion, with no LH surge or subsequent rise in serum progesterone (Fraser et al. 2015a), suggesting the role of NKB in regulation of female reproduction. Follicle development was not assessed in this study, but antagonism of NK3R was associated with reduced ovarian weight and absence of normal cyclical changes seen in the uterine mucosa, the effects which were reversible upon discontinuation of NK3R antagonist (Fraser et al. 2015a). However, the role of NKB in regulating gonadotropin secretion and ovarian function in healthy women was then unknown, and this study was designed to determine this in premenopausal women.

During the preparation of this thesis, data on the effects of the NK3R antagonist ESN364 administered in healthy women for 21 days initiated early in the follicular phase were published (Fraser et al. 2016). NK3R antagonist in those women decreased estradiol secretion and affected the LH surge, which was variably delayed but not completely abolished (Fraser et al. 2016). As a result of the delayed LH surge, progesterone rise was inhibited and menstrual cycle length was also prolonged, although no significant changes in follicle development were observed (Fraser et al. 2016). The NK3R antagonist did not affect basal LH levels and changes

in GnRH/LH pulsatility were not investigated. The mechanisms by which NK3R antagonist regulates ovarian function therefore remain unclear. NK3R has been localised to human granulosa cells (Cejudo Roman et al. 2012, Garcia-Ortega et al. 2014, Garcia-Ortega et al. 2016). The effects of NK3R antagonism on LH pulsatility and ovarian follicle development was therefore determined in premenopausal women during the follicular phase of the menstrual cycle to investigate the possible contribution of NKB signalling to the regulation of follicle development.

5.2 Objectives

The objectives of this study were to investigate the effect of NK3R antagonism in the follicular phase in healthy women on:

1) gonadotropin and ovarian hormone secretion;
2) LH pulsatility;
3) follicle development, and the endometrial response.

5.3 Methods

5.3.1 Participants

Thirteen healthy premenopausal women, aged 27-41 years and with regular menstrual cycles (25-34 days) based on medical history were recruited into the study; all volunteers provided informed written consent. Subjects were not taking any hormonal contraception nor had a copper intrauterine device in situ. They were eligible based on inclusion and exclusion criteria (Table 2.1 Inclusion and exclusion criteria, Chapter 2), had normal physical examination and negative urine pregnancy test. Full blood count, renal function, electrolytes, liver function and electrocardiogram were within normal limits.

5.3.2 Study drugs

The specific neurokinin-3 receptor (NK3R) inhibitor AZD4901 was administered orally at 40 mg twice daily.

5.3.3 Protocol

Investigation of the effect of NK3R antagonism on gonadotropin and ovarian hormone secretion.

Schematic presentation of the protocol is shown in Figure 5.1. Volunteers were administered the NK3R antagonist for 7 days starting on cycle day 5-6. All women had a no treatment control cycle, and a treatment cycle, the order of cycles being randomised. Peripheral venous blood was sampled for spot LH, FSH, inhibin B and estradiol immediately before treatment (pre-treatment), on days 2, 4 and 6 of NK3R antagonist administration, in the morning following the last dose and then every 2-4 days until ovulation was confirmed by transvaginal ultrasonography. Ovulation was defined either by the last day on which the pre-ovulatory follicle was seen or appearance of a corpus luteum. In the control cycle interval blood sample timing was equivalent to that in the treatment cycle. Once daily blood sampling was performed in the morning: during treatment, this was immediately prior to the next dose of NK3R antagonist, i.e. 12 hours after the previous dose. Urine was collected daily for progesterone until the next menstrual period.

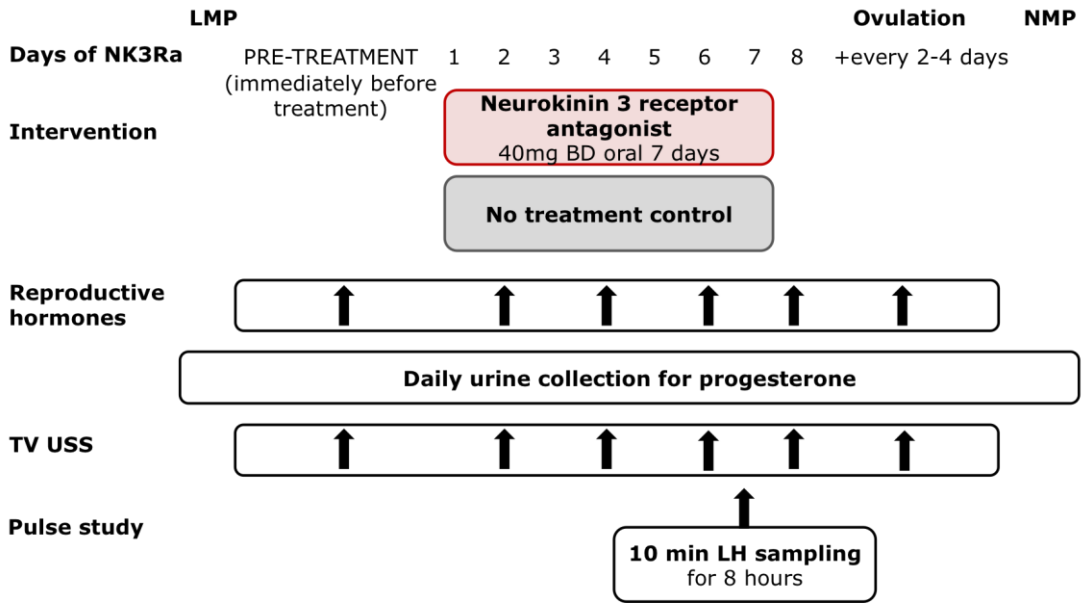


Figure 5.1 Study protocol.

13 healthy women were administered the NK3R antagonist AZD4901 orally for 7 days from cycle day 5-6. Reproductive hormones were measured and transvaginal ultrasonography was performed throughout the study. Urine samples were collected daily until the next menstrual period. LH pulsatility (n=8) was assessed during 8 hours of 10 minute blood sampling on day 6 or 7 of NK3R antagonist administration or equivalent day of the control cycle. Reproductive hormones and ultrasound scan findings were compared to the control cycle, the order of cycles being randomised. LMP, last menstrual period; NMP, next menstrual period; TV USS, transvaginal ultrasonography; BD, twice daily.

Investigation of the effect of NK3R antagonism on follicle development and endometrial thickness.

Transvaginal ultrasonography was used to measure the diameter of the leading follicle, any follicles of ≥10 mm in diameter, endometrial thickness and appearance of corpus luteum. Ultrasound scans were performed with the same schedule as assessment of reproductive hormones.

Investigation of the effect of NK3R antagonism on LH pulsatility.

Assessment of LH pulsatility was performed in 8 of the 13 women. On day 6 or 7 of NK3R antagonist treatment volunteers attended the clinical research facility for 8 hours. All visits commenced between 0800 and 0900 hours to avoid diurnal variation. Blood samples were collected via an indwelling intravenous cannula at 10 min intervals for 8 hours. The dose of NK3R antagonist was administered

immediately prior to the start of sample collection. In control cycle, frequent sampling was performed on cycle day 10-12, i.e. on the equivalent cycle day to that in the treatment cycle.

Safety profile.

Safety blood tests including full blood count, renal function and electrolytes, and liver function were checked before commencing the NK3R antagonist, after the treatment had finished and 2-3 weeks later.

5.3.4 Analytical methods

Hormone assays, analysis of pulsatile LH secretion and safety blood tests were performed as described in Chapter 2.

5.3.5 Statistical analysis

Data were assessed for Gaussian distribution by Shapiro-Wilk normality test (for data points ≥7) or Kolmogorov-Smirov test as appropriate (for data points <7). Baseline characteristics between the control and NK3R antagonist-treated cycles were compared by Student's paired t-test (for normally distributed data: LH, FSH, estradiol, follicle size) or Wilcoxon matched-pairs signed rank test (for data that did not have a normal distribution: menstrual cycle day). Hormone concentrations and ultrasonography data were compared throughout 7 days of NK3R antagonist treatment and between the control and treatment groups using repeated measure two-way ANOVA followed by Bonferroni's multiple comparisons post hoc analysis. Peak serum hormone concentration, size of the pre-ovulatory follicle and their peak cycle timing were compared by Student's paired t-test. Urinary progesterone concentrations, normalised to creatinine concentrations, were available for 11 women. Mid-luteal (i.e. LH surge +7 days) urinary progesterone levels and cycle length were compared by Student's t-test. Characteristics of pulsatile LH secretion were compared in 8 subjects by paired Student's t-test. Data are presented as mean ± SEM.

5.4 Results

5.4.1 Baseline characteristics

Each subject took part in the control and treatment cycle, which were comparable by cycle day on which the study had started, baseline serum LH, FSH and estradiol concentrations, and the size of the largest ovarian follicle present at that time (Table 5.1).

Characteristic	Control cycle	NK3Ra cycle	p value
Cycle day study commenced	5.6 ± 0.2	5.0 ± 0.2	0.06
LH (IU)	4.9 ± 0.5	4.8 ± 0.4	0.98
FSH (IU)	5.1 ± 0.8	5.3 ± 0.9	0.82
Estradiol (pmol/l)	160 ± 19	126 ± 13	0.07
Follicle diameter (mm)	8.4 ± 0.4	8.1 ± 0.3	0.54

Table 5.1 Basic characteristics of the participants.

5.4.2 NK3R antagonist does not affect basal LH secretion but delays mid-cycle LH surge

NK3R antagonist had no effect on LH secretion during single timepoint analysis (Fig. 5.2A) and 8 hours of frequent LH sampling every 10 minutes (Fig. 5.2E). However, the LH surge was significantly delayed in the NK3R antagonist cycle by 7 days, i.e. the duration of treatment (cycle day: 22±1 vs 15±1, p=0.0006) (Fig. 5.2A and 5.6A). There was no effect on the magnitude of the peak of mid-cycle LH secretion (23.4±4.8 with NK3R antagonist vs 19.7±3.2 IU/l, ns [data not shown]).

5.4.3 NK3R antagonist has differential effect on FSH secretion

FSH secretion was unchanged with the NK3R antagonist on single day sampling (Fig. 5.2B). To detect subtle changes in hormone secretion over time potentially overlooked by performing single time spot blood sampling, a more detailed analysis of FSH secretion every hour for 8 hours post NK3R antagonist dose showed an increase in FSH secretion throughout the 8 hour-period with the NK3R antagonist administration compared to control cycle (ANOVA $p<0.03$, Fig. 5.2F) with FSH concentrations being higher at each hour sampled ($p<0.05$ at every hour, Fig. 5.2F).

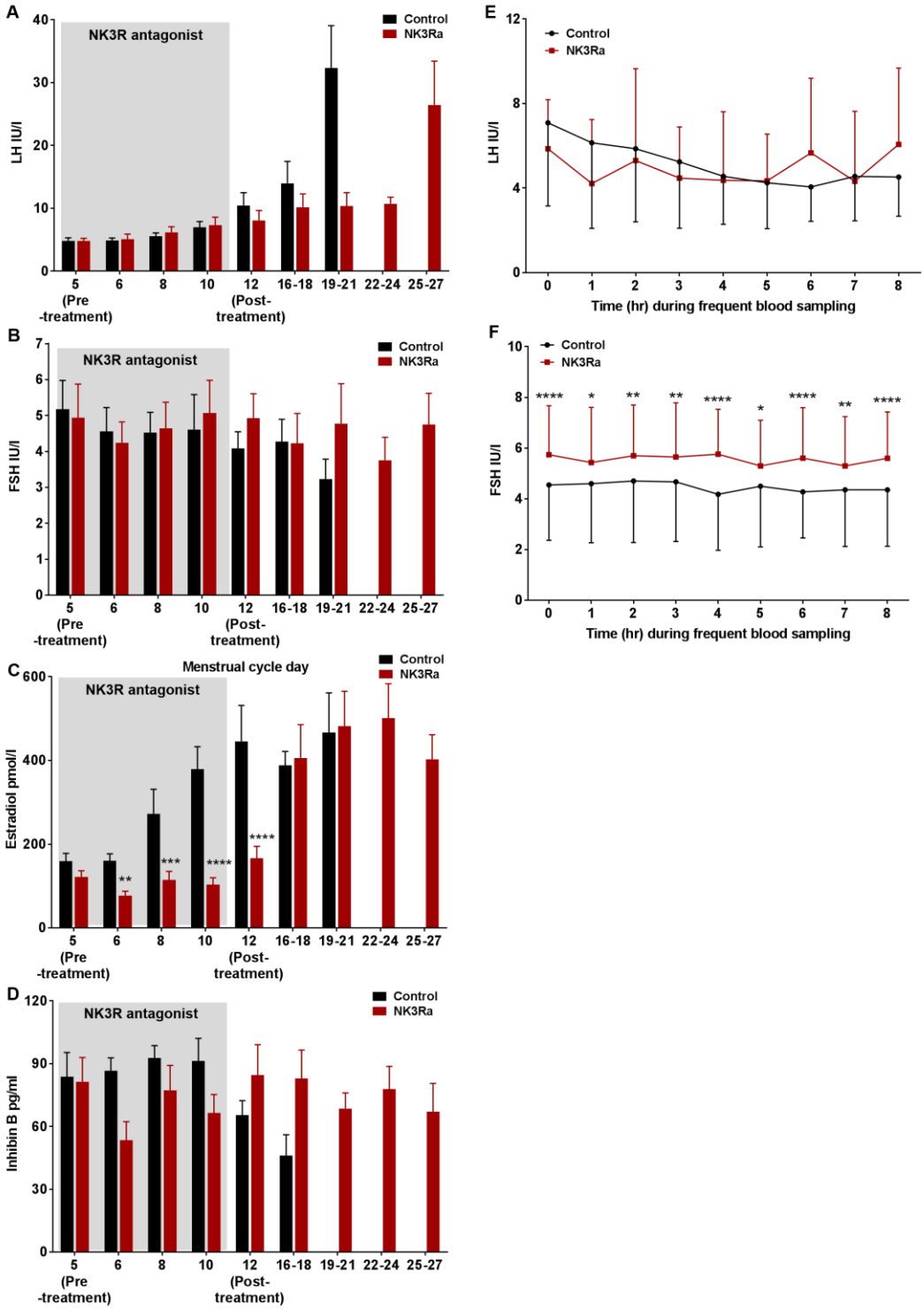

A
NK3R antagonist
Control
NK3Ra
LH IU/l
5 (Pre -treatment)
6
8
10
12 (Post-treatment)
16-18
19-21
22-24
25-27
B
FSH IU/l
C
Menstrual cycle day
Estradiol pmol/l
D
Inhibin B pg/ml
E
Time (hr) during frequent blood sampling
F

Figure 5.2 Reproductive hormone response in premenopausal women in the control and NK3R antagonist-treated cycles.

Mean serum LH **(A)**, FSH **(B)**, estradiol **(C)** and inhibin B secretion **(D)** with and without NK3R antagonist was compared in premenopausal women (n=13) by repeated measure two-way ANOVA at timepoints when paired data was available (i.e. between cycle days 5-16/18 inclusive) followed by Bonferroni's multiple comparisons post hoc analysis. **(E)** LH concentrations during frequent every 10 minute blood sampling and **(F)** FSH concentrations during hourly blood sampling for 8 hours post NK3R antagonist administration on day 6-7 compared to no treatment control cycle in premenopausal women (n=8). Data are presented as mean ± SEM. *, $p<0.05$ **, $p<0.01$; ***, $p<0.001$; ****, $p<00001$.

5.4.4 NK3R antagonist delays estradiol rise, which resumes after stopping the treatment

Estradiol concentration was significantly lower at each timepoint throughout treatment ($p<0.05$ vs pre-treatment) (Fig. 5.2C). At the end of NK3R antagonist administration (166±29 vs control 446±86 pmol/l day 12, $p<0.0001$), estradiol concentrations remained similar to baseline levels on cycle day 5 (126±16 pmol/l, ns). After NK3R antagonist treatment, estradiol concentrations rose, reaching pre-ovulatory levels comparable to those in control cycles (690±68 with NK3R antagonist vs 699±62 pmol/l, ns [data not shown]) but 7 days later (cycle day 21±1 vs 14±1, $p=0.002$) (Fig. 5.6B).

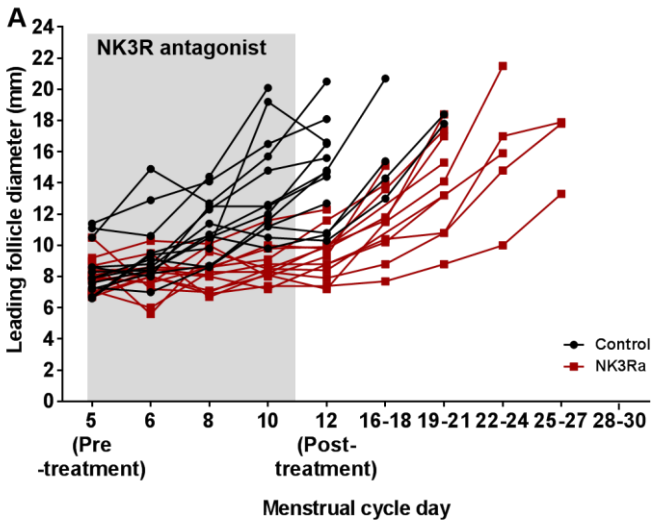
5.4.5 NK3R antagonist effects on inhibin B secretion

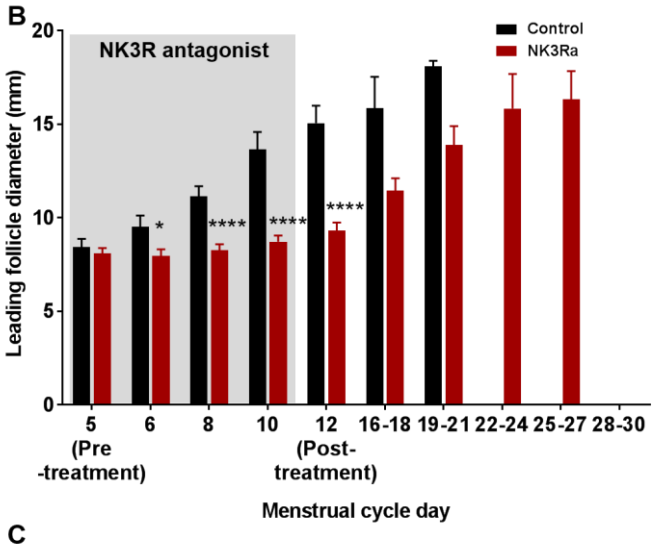
Inhibin B secretion (a marker of small follicle development) was slightly reduced during NK3R antagonist administration, but this did not reach statistical significance (Fig. 5.2D).

5.4.6 NK3R antagonist delays follicle growth, which resumes after stopping the treatment

Follicle growth was suppressed and delayed in the NK3R antagonist treatment cycles, closely matching the changes in estradiol secretion. Figure 5.3A shows follicle growth until ovulation for each subject in the control and NK3R antagonist-treated cycle, with mean data in Figure 5.3B. While there was a progressive rise in diameter of the leading follicle in control cycles, this did not occur during 7 days of NK3R antagonist treatment (ANOVA $p=0.0003$). The diameter of the leading follicle was significantly smaller than in controls at each timepoint throughout 7 days of treatment ($p<0.05$ vs pre-treatment) and at the end of NK3R antagonist i.e. on cycle day 12 (9.3±0.4 vs 15.1±0.9 mm, $p<0.0001$) (Fig. 5.3B). After treatment normal follicle development resumed, reaching the same pre-ovulatory follicle size as in controls (16.1±0.7 with NK3R antagonist vs 17.2±0.7 mm, ns) but later (cycle day: 21±1 vs 15±1, $p=0.002$) (Fig. 5.6C).

NK3R antagonist delayed ovulation in 11 of 13 subjects. One subject had earlier ovulation with NK3R antagonist compared to her control cycle and one subject ovulated on the same day with or without the NK3R antagonist treatment (Fig. 5.3C).





C

Ovulation (n)	Menstrual cycle day	12	16-18	19-21	22-24	25-27	28-30
	Control	1	8	2	2		
	NK3Ra		1	2	3	4	3

Figure 5.3 Follicle development in 13 premenopausal women in the control and NK3R antagonist-treated cycle until ovulation.

(A) Follicle growth in each of the 13 premenopausal women showing delayed development with the NK3R antagonist treatment (*red squares*) compared to the control cycle (black circles). **(B)** Mean follicle diameter in the control and NK3R antagonist-treated cycle. Data include all cycles where the leading follicle remained i.e. only include women who had not yet ovulated at the later time points. **(C)** Table showing the timepoint in the control and NK3R antagonist cycle at which the leading follicle was no longer identified at transvaginal ultrasonography. Data were compared by repeated measure two-way ANOVA at timepoints when paired data was available (i.e. between cycle days 5-12 inclusive) followed by Bonferroni's multiple comparisons post hoc analysis. Data are presented as mean ± SEM. *, $p<0.05$; ****, $p<0.0001$.

5.4.7 NK3R antagonist delays endometrial development

Endometrial development was also affected by the NK3R antagonist treatment ($p<0.0001$), being significantly thinner than in controls at the end of treatment (4.6±0.4 vs 7.7±0.5 mm day 12, $p<0.0001$) (Fig. 5.4). Thereafter endometrial thickness increased, reaching a similar thickness to that in control cycles at the time of ovulation (8.9±0.6 vs 9.5±1.0 mm, ns).

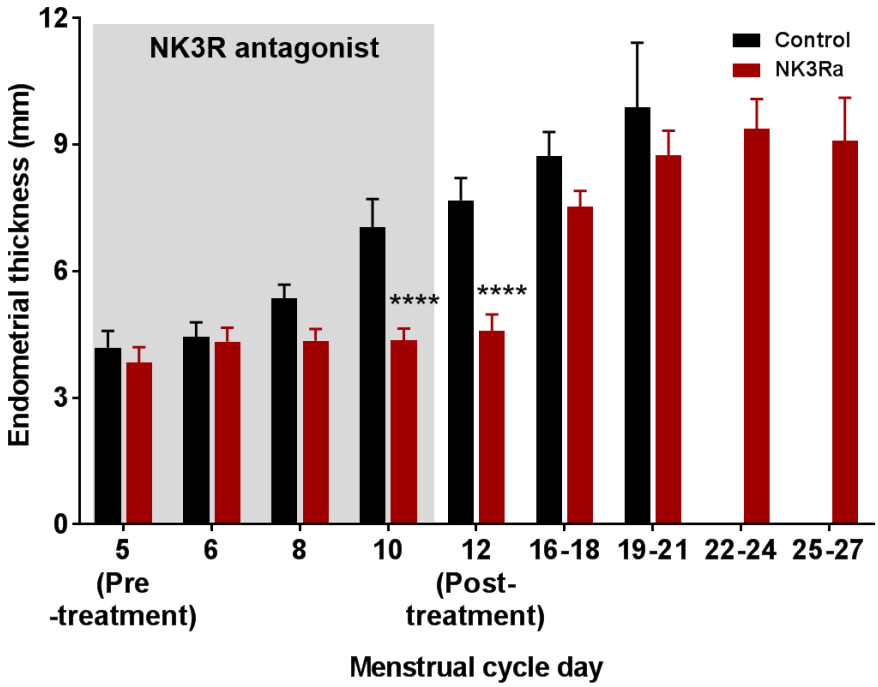


Figure 5.4 Endometrial development in 13 premenopausal women in the control and NK3R antagonist-treated cycle.

NK3R antagonist was administered from average cycle day 5 for 7 days with the last treatment day on average cycle day 11. Mean endometrial thickness was compared by repeated measure two-way ANOVA at timepoints when paired data was available (i.e. between cycle days 5-16/18 inclusive) followed by Bonferroni's multiple comparisons post hoc analysis. Data are presented as mean ± SEM. ****, $p<0.0001$.

5.4.8 NK3R antagonist delays luteal progesterone rise

Consistent with the above demonstration of delayed ovulation, NK3R antagonist delayed the cycle day of mid-luteal progesterone (cycle day LH surge +7: 30±2 vs 22±1, p=0.002) (Fig. 5.6D). However, when standardised against the day of the LH surge, luteal function was not affected by the NK3R antagonist (urinary progesterone 58±10 vs 48±7 pmol/mol creatinine on LH surge day +7, ns) (Fig. 5.5).

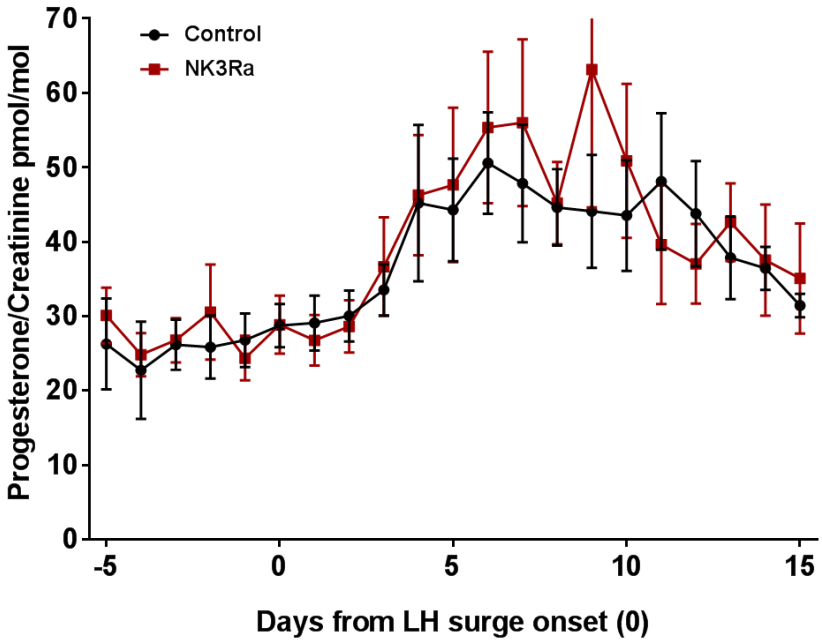


Figure 5.5 Mean urinary progesterone/creatinine ratio with and without NK3R antagonist in 11 premenopausal women adjusted to LH surge onset day 0.

5.4.9 NK3R antagonist prolongs menstrual cycle length

Menstrual cycle length was prolonged by approximately 6 days with the NK3R antagonist further supporting delayed ovulation in the treatment group (35±1 vs 29±1 days, p=0.0003) (Fig. 5.6E).

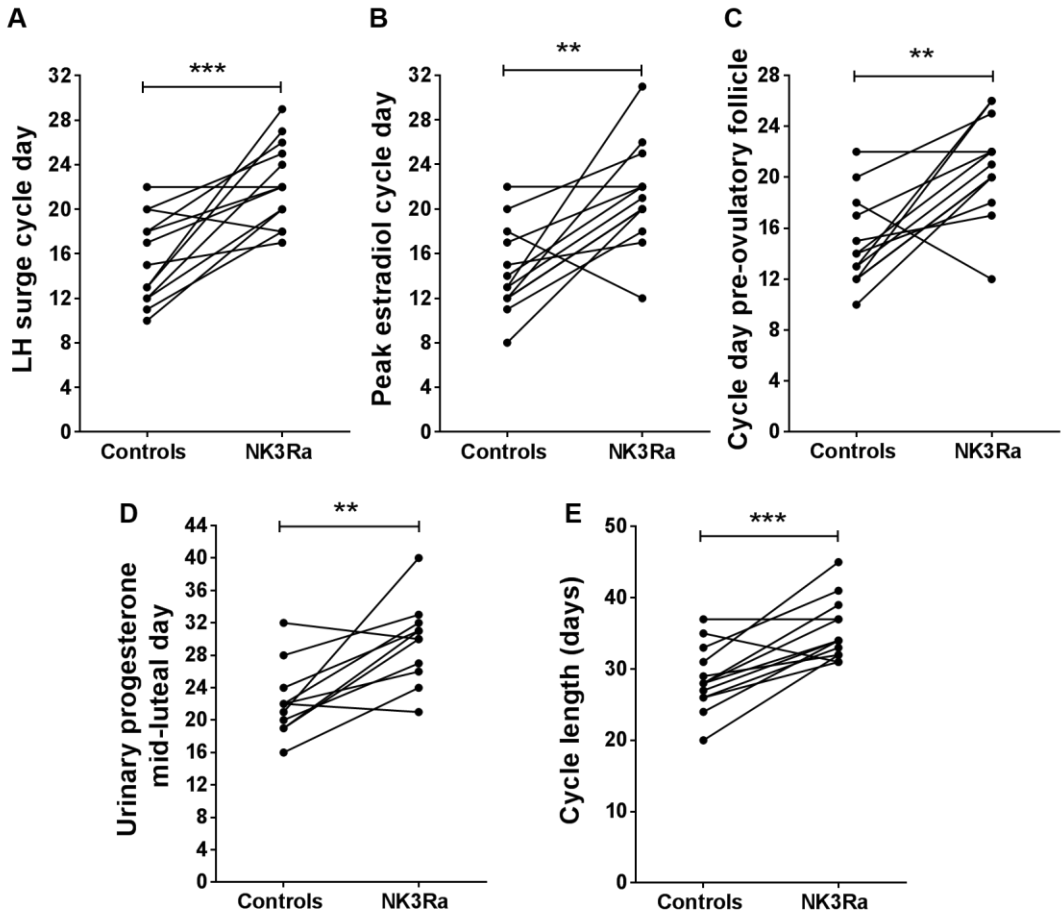


Figure 5.6 Summary of the delay in timing of key events in female reproduction with the NK3R antagonist compared to no treatment control cycle in premenopausal women.

Individual subject (n=13) response to NK3R antagonist showing delay in menstrual cycle day for LH surge **(A),** peak estradiol **(B)**, appearance of the largest diameter of the pre-ovulatory follicle **(C)**, mid-luteal urinary progesterone (day at surge +7, n=11) **(D)**, and the length of menstrual cycle **(E)**. Note that two subjects in figure **(B)** and **(C)** had the same cycle days for the events described. **, $p<0.01$; ***, $p<0.001$.

5.4.10 NK3R antagonist effects on pulsatile LH secretion

Assessment of pulsatile LH secretion was performed in 8 of the 13 women on day 6 or 7 of NK3R antagonist treatment and on the equivalent day of the control cycle. An example of an LH pulse frequency profile is shown in Figure 5.7A. Although LH pulse frequency did not change with the NK3R antagonist (0.69±0.1 vs 0.66±0.1 pulses/hr, ns), basal (i.e. nonpulsatile) LH secretion was reduced ($p<0.05$) (Fig. 5.7B and C). NK3R antagonist had no overall effect on secretory mass of LH per pulse (Fig. 5.7D) and total amount of LH secreted in a pulsatile manner (Fig. 5.7E). A slight improvement in the orderliness of LH secretory pattern approached statistical significance ($p=0.054$) (Fig. 5.7F).

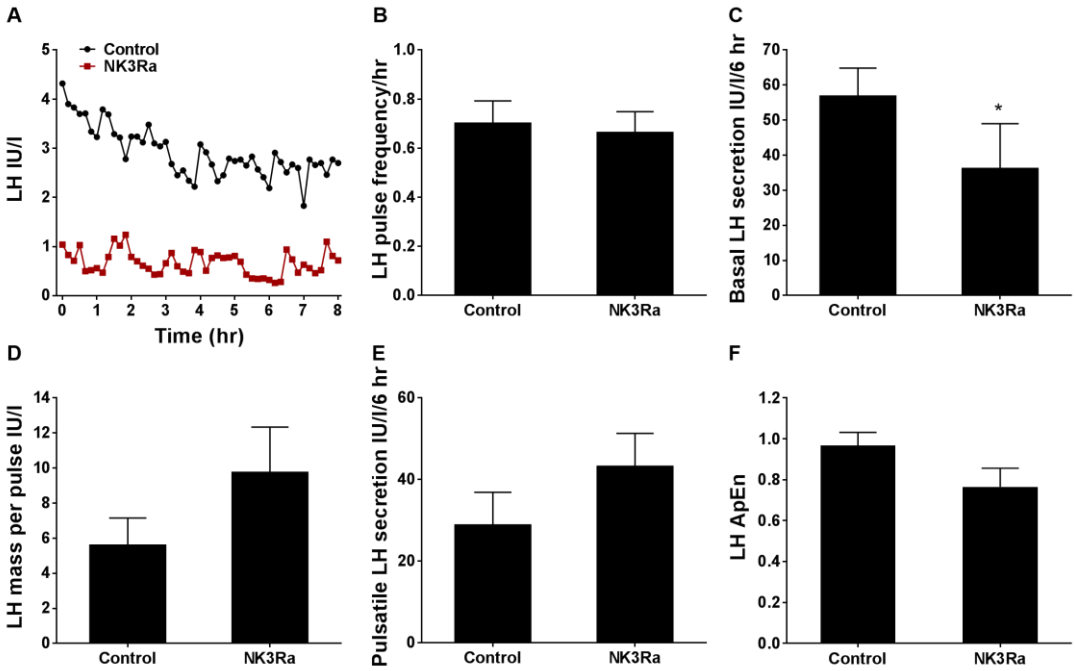


Figure 5.7 Pulsatile LH secretion in premenopausal women in the control and NK3R antagonist-treated cycle.

Illustrative LH pulse profile from one subject **(A)** undergoing 10 minute blood sampling for LH for 8 hour with no NK3R antagonist (*black circles*) and on day 7 of NK3R antagonist treatment (*red squares*). Mean LH pulse frequency **(B)**, basal (nonpulsatile) LH secretion **(C)**, mass of LH per pulse **(D),** pulsatile LH secretion **(E)** and relative orderliness/regularity of LH secretory pattern **(F)** were compared between the control and NK3R antagonist-treated cycles (n=8). Data are presented as mean ± SEM. *, $p<0.05$.

5.4.11 Tolerability and safety

NK3R antagonist was well tolerated with no treatment discontinuations. No subjects reported menopausal symptoms whilst on NK3R antagonist, with estradiol remaining above 100 pmol/l. Haematology and biochemistry safety parameters remained stable in all subjects throughout the study period. All subjects returned to their usual menstrual cycle length following NK3R antagonist-treated cycle.

5.5 Discussion

This study has investigated the role of neurokinin B in regulating physiological follicle development and its hypothalamic action through the modulation of pulsatile GnRH/LH secretion in premenopausal women. NK3R antagonism for 7 days in the early follicular phase in healthy women suppressed follicle growth and estradiol secretion and delayed ovulation by the duration of treatment. Deconvolutional analysis of LH secretion showed a reduction of basal LH secretion, without a change in pulse frequency, whereas single timepoint analysis did not show an effect on LH release, probably because this was 12 hours after the previous NK3R antagonist dose. The half-life of AZD4901 is approximately 8.5 hours (Litman et al. 2014). However, FSH secretion was increased with the NK3R antagonist when analysed during frequent sampling over 8 hours post dosing. Taken together, this suggests that selective blockade of NK3R reduces follicular estradiol production and arrests follicle growth by reducing basal GnRH/LH secretion, and this effect persisted for the duration of treatment. These findings confirm an important role of NKB in human reproduction and provide clear evidence for NKB signaling in the physiological regulation of normal follicle development in women.

A further striking finding was that the effect of NK3R antagonist were reversible after discontinuation of treatment, with normal follicle estradiol production and growth resuming, resulting in a normal LH surge and mid-luteal progesterone rise, all of which were delayed by the duration of treatment. This indicates that growth of the dominant follicle can be suspended by manipulation of the endocrine environment without atresia being inevitable, although oocyte competence for fertilisation was not tested.

The decreased estradiol secretion for the duration of 7-day treatment in healthy women was biologically relevant as shown by absent development of the endometrium during NK3R antagonist treatment, with later growth as follicle estradiol production increased after drug discontinuation. A recent study using a different NK3R antagonist (ESN364) in normal women for 21 days throughout the follicular phase failed to detect any significant suppression in follicle growth, despite other findings being consistent with our data, particularly a delayed LH surge in some women and prolongation of menstrual cycle length (Fraser et al. 2016). It is likely that the more variable effect on LH surge possibly due to ESN364 being less potent suppressor of basal LH secretion than AZD4901 used here precluded clear demonstration of an effect of folliculogenesis. While antral follicle development was clearly delayed by treatment with the NK3R antagonist in this study, there was not clear evidence of an effect on the growth of smaller follicles, as inhibin B levels were not significantly reduced. However, the markedly suppressed estradiol is likely to contribute to the increase in FSH secretion observed with the NK3R antagonist. FSH secretion is also promoted (relative to LH) by basal rather than pulsatile GnRH secretion (McNeilly et al. 2003), thus two mechanisms may contribute to the raised FSH concentrations observed.

The absence of detectable effects of NK3R antagonist on LH secretion in this study is consistent with previous data showing only a temporary suppression of LH levels lasting few hours post dosing, but no overall decrease in gonadotropin secretion with a different NK3R antagonist (ESN364) throughout the follicular phase in normal women (Fraser et al. 2016) and in intact female monkeys (Fraser et al. 2015a). These findings are similar to minimal suppression on LH secretion observed in postmenopausal women in Chapter 4 after 7 days administration of the same dose of NK3R antagonist used here. However, marked decrease in LH secretion with the NK3R antagonist was shown in ovariectomised ewes (MRK-08) (Li et al. 2015) and castrate monkeys (ESN364) (Fraser et al. 2015a), although treatment was administered centrally and in much higher concentrations, respectively. It is possible that loss of negative feedback with decreased serum estradiol in this study limited the ability of NK3R antagonist to suppress LH secretion. Duration and/or dose of NK3R

antagonist and sampling regimen acknowledged above may have contributed to the lack of effects seen on LH secretion, especially as patients with loss-of-function mutations in NKB and its cognate receptor exhibit a significant phenotype of hypogonadotropic pubertal delay (Topaloglu et al. 2009). Ovulation was delayed in 11 of the 13 women with two women being non-responders. This suggests that the dose and regimen used in this study may be at the bottom of the dose-response curve. It is possible that with higher doses of NK3R antagonist, a more marked inhibitory action on LH secretion would be observed. Nevertheless, consistent with the effects on pulsatile LH secretion, antagonism of NK3R resulted in marked ovarian effects: follicle maturation and ovarian hormone secretion were delayed, postponing the LH surge and subsequent luteal progesterone rise. The lower serum estradiol levels with the NK3R antagonist suggests that LH dependent secretion of thecal androgens was suppressed. Granulosa cell proliferation (and thus follicle growth) is predominantly driven by FSH, which was not suppressed but rather increased by the NK3R antagonist, an observation not reported in the previous studies. It may be that NK3R antagonist therefore exerts direct effects on granulosa cells (Cejudo Roman et al. 2012, Garcia-Ortega et al. 2014, Garcia-Ortega et al. 2016). Reduction in ovarian weight was reported with 35-day administration of NK3R antagonist in intact female monkeys, although this was not associated with any histopathological findings in the ovary (Fraser et al. 2015a). Stromal atrophy and glandular inactivity in the uterine mucosa of those monkeys related to the NK3R antagonist were reversible upon cessation of drug treatment (Fraser et al. 2015a). This is consistent with the return of pre-treatment ovarian function and menstrual cycle length in women in this study after the NK3R antagonist has prolonged it. Delay in key events in female reproductive cycle for the duration of NK3R antagonist administration, which was 7 days in this study, lends further support for temporary treatment effects.

The present study has for the first time investigated a hypothalamic mode of action of NKB in healthy women, showing that in the follicular phase NK3R antagonist reduced basal i.e. nonpulsatile LH and by inference GnRH secretion with no effect on pulse frequency. Marked suppression in LH secretion between the pulses is consistent with data from men and postmenopausal women shown in Chapter 3 and

Chapter 4, respectively. Changes in basal LH/GnRH secretion with no effect on pulse frequency in these studies were sufficient to decease LH secretion in postmenopausal women and LH and testosterone in men, and in premenopausal women suppressed ovarian follicle estradiol production and growth and delayed ovulation. This suggests that NKB signalling may be integral in regulating basal LH/GnRH secretion, and that the frequency of LH pulses is predominantly controlled by another hypothalamic peptide, such as kisspeptin (George et al. 2011, Young et al. 2013). In men and postmenopausal women, other parameters of LH secretory pattern were affected by the NK3R antagonist, including mass of LH secreted per pulse, pulsatile LH release and ApEn. Reduction in the frequency of LH/GnRH pulses by the NK3R antagonist has been demonstrated previously but only in state of high LH output, such as in women with PCOS (George et al. 2016) and in gonadectomised animals (Fraser et al. 2015a, Li et al. 2015). Patients with inactivating mutations in NKB pathway had diminished LH pulse frequency (Young et al. 2013) and therefore it remains possible that with larger doses of NK3R antagonist such effect would be observed in healthy women. There are no other published data to compare the effect of NKB antagonism on LH/GnRH pulsatility in health, and the only study administering NKB itself as an infusion over 8 hours in men showed no effect on any parameters of pulsatile LH secretion (Jayasena et al. 2014c). Taken together these data suggest that downstream ovarian effects of NK3R antagonist in premenopausal women are mediated via modulation of basal GnRH/LH secretion.

The suppressive effect of NK3R antagonist on nonpulsatile GnRH/LH but increased FSH release allows ongoing estradiol secretion from granulosa cells with estradiol levels remaining above 100 pmol/l in this study. This alleviates concerns of unwanted menopausal-like side effects, which are associated with the clinical use of GnRH analogues, whereby blockade of GnRH receptor downregulates the HPG axis with more marked suppression of estradiol secretion (Maggi et al. 2016). There were no reports of hot flushes in premenopausal women whilst taking the NK3R antagonist in this study. Selective blockage of NKB signalling might therefore have therapeutic potential in the management of sex-steroid disorders, such as

endometriosis, fibroids and heavy menstrual bleeding. NK3R antagonist as a novel non-steroidal contraceptive is also a possible indication. Although follicle growth was arrested during 7 days of NK3R antagonist treatment, it remains unclear if such effect would persist with longer use and might be limited by lack of progesterone exposure and adverse endometrial outcomes.

In summary, NK3R antagonism in healthy premenopausal women in the early follicular phase of menstrual cycle, supressed follicle growth and estradiol secretion and delayed ovulation for the duration of treatment. Those effects were reversible after cessation of drug administration with evidence of normal ovulation and luteal function. Whilst NK3R antagonist had no effect on LH secretion (by once-daily sampling), detailed analysis revealed a marked effect on basal LH secretion in normal women, thus providing strong support for the role of NKB signalling in modulating GnRH secretion. These data confirm the involvement of NKB in the physiological neuroendocrine control of female reproduction. Our studies suggests that blockade of NKB-NK3R signalling may have potential therapeutic application in non-steroidal contraception and in the management of sex-steroid dependent disorders, e.g. endometriosis, fibroids, prostatic hypertrophy (see section 8.5 Clinical application of NKB-KP manipulation and future work).

Chapter 6. Neurokinin B in the regulation of ovarian function in healthy women in the late follicular and luteal phases of menstrual cycle

6.1 Introduction

In Chapter 5, the role of NKB in regulating reproductive hormone secretion and ovarian follicle development in healthy women was investigated. When administered in the early follicular phase, NK3R antagonist (AZD4901) prevented ovarian follicle development and estradiol secretion and delayed ovulation for the duration of treatment. Those effects were reversible upon discontinuation of treatment. This is consistent with recently published data using a different NK3R antagonist (ESN364), showing delayed pre-ovulatory estradiol rise and variable effects on the LH surge with delayed mid-luteal progesterone secretion over 21 day administration in women (Fraser et al. 2016) and similar findings in rhesus monkeys (Fraser et al. 2015a). The mechanism of action, or at least a part of it, by which NK3R antagonism achieved those effects in normal women appears to be by decreasing basal, non-pulsatile LH secretion through presumed similar changes in GnRH secretion. These data clearly supports a role for NKB in modulating GnRH secretion in the early follicular phase in female reproduction. However, in those studies effects of NK3R antagonism later in the cycle reflect suppressed estradiol secretion in the early follicular phase. There are no studies investigating the role of NKB signalling in a different sex-steroid environment, specifically when administered in the late follicular or luteal phases in normal women.

The involvement of NKB in the mid-cycle LH surge has been suggested in animal models (Billings et al. 2010, Navarro et al. 2011a, Ramaswamy et al. 2011). In ewes, the NK3R agonist senktide stimulated LH secretion to levels mimicking a surge, whilst the NK3R antagonist reduced LH surge amplitude (Billings et al. 2010). However, these results have not been reproduced in other species, including humans. Recent evidence suggest that NKB is not a prerequisite for positive estrogen feedback since centrally administered NK3R antagonist (MRK-08) failed to abolish estradiol-induced LH surge in an ovariectomised sheep, although the onset to peak

time of the LH surge was delayed (Li et al. 2015). Unlike an upregulation of *Kiss1* at the pre-ovulatory LH surge in ewes (Smith et al. 2006b), the expression of *Tac3* did not change in ovariectomised ewes in response to a surge-inducing estrogen challenge (Goubillon et al. 2000). In the studies in Chapter 5 of NK3R antagonist administration from the early follicular phase, the LH surge was delayed secondary to reduced estradiol secretion from arrested follicle growth. Whether blockade of NK3R antagonism would also suppress the development of a dominant ovarian follicle in the late follicular phase and subsequently affect sex-steroid secretion and the LH surge remains unknown.

The role of NKB in regulating the function of the corpus luteum has not yet been specifically explored. After ovulation, marked increase in progesterone levels exert a profound negative feedback slowing the frequency of GnRH pulses and thus LH secretion in the late luteal phase and favouring the rise in FSH necessary to initiate the next cycle (Yen et al. 1972, McNeilly et al. 2003). We have shown that NK3R antagonist administered early in the cycle postponed mid-luteal progesterone secretion, but it affected neither luteal function, nor its duration, consistent with the delayed ovulation observed with treatment. Similarly, delayed ovulation and luteal progesterone secretion were observed in healthy women with 21-day administration of ESN364 (Fraser et al. 2016). In intact female monkeys, mid-luteal progesterone rise was attenuated by the NK3R antagonist, but treatment was initiated in the early follicular phase and throughout the luteal phase for 35 days, and the results are consistent with overall suppression of pre-ovulatory estradiol secretion and hence the LH surge (Fraser et al. 2015a). Effects of NK3R antagonist on endometrium were of stromal atrophy and glandular inactivity (Fraser et al. 2015a). Menstrual cycle and uterine mucosa changes were reversible in those animals after discontinuation of treatment (Fraser et al. 2015a). All together, these data suggest that when administered in the early follicular phase NK3R antagonist is able to prevent or delay the normal events of the menstrual cycle, but it remains to be explored if antagonism of NKB-NK3R pathway would interrupt the progression through the menstrual cycle when administered in the late follicular phase or affect luteolysis with treatment initiated following ovulation.

6.2 Objectives

The objectives of this study were to investigate the effect of NK3R antagonism in healthy women during:

1) the late follicular phase, on gonadotropin and ovarian hormone secretion, follicle development and the endometrial response;
2) the luteal phase, on gonadotropin secretion, luteal function and endometrial thickness.

6.3 Methods

6.3.1 Participants

Twelve healthy premenopausal women (eight women being the same participants as in previous study), aged 27-45 years and with regular menstrual cycles (26-34 days) based on medical history were recruited into the study; all volunteers provided informed written consent. Subjects were not taking any hormonal contraception nor had a copper intrauterine device in situ. They were eligible based on inclusion and exclusion criteria (Table 2.1 Inclusion and exclusion criteria, Chapter 2), had normal physical examination and negative urine pregnancy test. Full blood count, renal function, electrolytes, liver function and electrocardiogram were within normal limits.

6.3.2 Study drugs

The specific neurokinin-3 receptor (NK3R) inhibitor AZD4901 was administered orally at 40 mg twice daily.

6.3.3 Protocol

Study 1: Effects of NK3R antagonism on gonadotropin secretion and ovarian function in the late follicular phase.

Schematic presentation of the protocol is shown in Figure 6.1A. Six subjects were administered the NK3R antagonist for 7 days from the day of emergence of a dominant (≥12 mm diameter) follicle determined by transvaginal ultrasonography performed on alternate days from cycle day 10. All women had a no treatment control cycle and a treatment cycle, the order of cycles being randomised. Peripheral

venous blood was drawn for spot LH, FSH, estradiol and progesterone assay, and transvaginal ultrasonography was performed for the leading follicle diameter and endometrial thickness immediately before treatment (pre-treatment), on days 2, 4 and 6 of NK3R antagonist administration, in the morning following the last dose and then 6-8 days later. In the control cycle interval timing for blood sampling and radiological assessment was equivalent to that in the treatment cycle. Study visits took place in the morning: during treatment, this was immediately prior to the next dose of NK3R antagonist, i.e. 12 hours after the previous dose.

Study 2: Effects of NK3R antagonism on gonadotropin secretion in the luteal phase and luteolysis.

Schematic presentation of the protocol is shown in Figure 6.1B. Six subjects were administered the NK3R antagonist for 7 days from day 2-3 after ovulation, which was defined as either by the disappearance of the dominant follicle or the appearance of a corpus luteum at transvaginal ultrasonography performed on alternate days from cycle day 12. All women had a no treatment control cycle and a treatment cycle, the order of cycles being randomised. Peripheral venous blood was drawn for spot LH, FSH, estradiol and progesterone assay immediately before treatment (pre-treatment), on days 2, 4 and 6 of NK3R antagonist administration and in the morning following the last dose. Endometrial thickness was assessed by transvaginal ultrasonography on the same days as interval blood sample timing. Blood sampling and radiological assessments were performed in the morning: during treatment, this was immediately prior to the next dose of NK3R antagonist, i.e. 12 hours after the previous dose.

Safety profile.

Safety blood tests including full blood count, renal function and electrolytes, and liver function were checked before commencing the NK3R antagonist, at the end of NK3R antagonist administration and 2-3 weeks later.

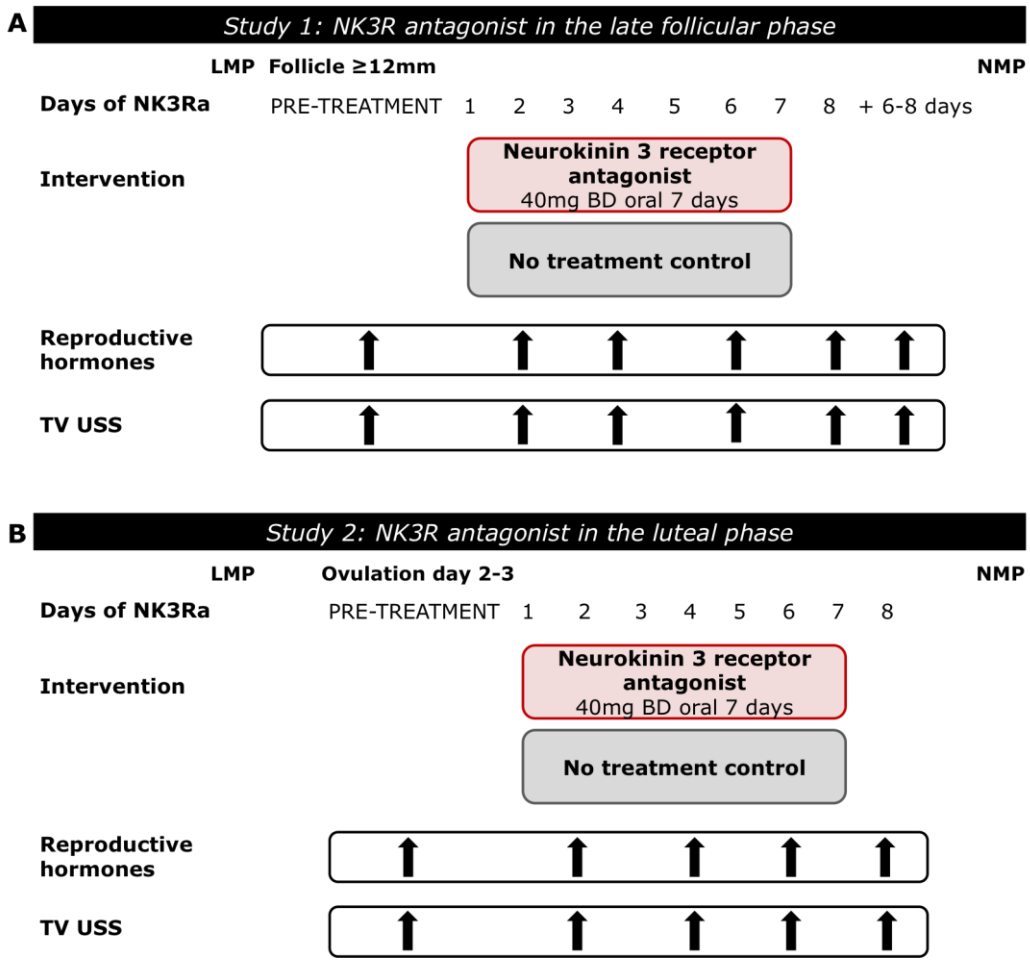


Figure 6.1 Study protocol.

(A) Study 1: NK3R antagonist AZD4901 was administered in healthy women (n=6) for 7 days in the late follicular phase following an emergence of a dominant ≥12 mm follicle. **(B)** Study 2: NK3R antagonist AZD4901 was administered in healthy women (n=6) for 7 days in the luteal phase from day 2-3 of ovulation. Reproductive hormones were measured and transvaginal ultrasonography was performed throughout the studies and compared to no treatment control cycle, the order of treatment and control cycles being randomised. LMP, last menstrual period; NMP, next menstrual period; TV USS, transvaginal ultrasonography; BD, twice daily.

6.3.4 Analytical methods

Hormone assays and safety blood tests were performed as described in Chapter 2.

6.3.5 Statistical analysis

Data were assessed for Gaussian distribution by Shapiro-Wilk normality test (for data points ≥7) or Kolmogorov-Smirnov test as appropriate (for data points <7). Baseline characteristics between the control and NK3R antagonist-treated cycles were compared by Student's paired t-test (for normally distributed data: follicle size, LH, FSH and estradiol) and by Wilcoxon matched-pairs signed rank test (for data that did not have a normal distribution: cycle days and progesterone).

Days to peak reproductive hormone concentrations and to the next menstrual period from the start of the study were compared by Wilcoxon matched-pairs signed rank test. The null hypothesis of equal variation in the number of days from the start of the study visits to the LH surge and peak FSH, estradiol and progesterone concentrations was tested by Levene's test for homogeneity of variance using statistical package system R, which was kindly performed by Dr Tom Kelsey at the University of St Andrews.

Mean hormone concentrations, follicle growth and the endometrial thickness between the control and NK3R antagonist-treated cycles were compared using repeated measure two-way ANOVA followed by Bonferroni's multiple comparisons post hoc analysis. The rate of follicle growth was calculated as change in millimetres since last measurement. Peak luteal estradiol and progesterone levels were compared by Student's paired t-test. Data are presented as mean ± SEM.

6.4 Results

6.4.1 Study 1: Effects of NK3R antagonist on gonadotropin secretion and ovarian function in the late follicular phase

6.4.1.1 Baseline characteristics

Table 6.1 shows that the control and NK3R antagonist-treated cycles were matched, with no pre-treatment differences in the diameter of the dominant ovarian follicle, serum reproductive hormones or cycle day on which the study commenced in the late follicular phase of menstrual cycle.

Characteristic	Control cycle	NK3Ra cycle	p value
Cycle day treatment commenced	13.7± 1.8	11.3 ± 1.2	0.09
LH (IU)	6.3 ± 1.3	7.0 ± 1.4	0.35
FSH (IU)	3.6 ± 0.4	3.6 ± 0.6	0.98
Estradiol (pmol/l)	393 ± 94	345 ± 62	0.70
Follicle diameter (mm)	12.9 ± 0.5	12.9 ± 0.2	0.95

Table 6.1 Basic characteristics of the participants in the late follicular phase of menstrual cycle. Control and NK3R antagonist-treated cycles were comparable at pre-treatment by the size of a dominant ovarian follicle, reproductive hormone levels and the menstrual cycle day on which the study visits had commenced (n=6). Data are presented as mean ± SEM.

6.4.1.2 NK3R antagonist effects on reproductive hormone secretion

All subjects showed an LH surge in both control and NK3R antagonist cycles. However, NK3R antagonist administration in the late follicular phase interfered with the timing of the LH surge, increasing the variation in the number of days from the emergence of a ≥12 mm follicle to the LH surge (Levene's test p=0.03) (Fig. 6.2A). The LH surge was earlier in three and later in another three subjects during NK3R antagonist treatment. There appeared some greater variation in time to peak estradiol concentrations preceding the LH surge, but this was not statistically significant (p=0.08), and no changes were observed in days to peak FSH secretion, or to subsequent peak progesterone concentrations (Fig. 6.2 B-D). There was no

difference in cycle length from the emergence of a dominant follicle to the onset of next menstrual period between the control and treatment cycle (NK3R antagonist: 18±1 vs 17±1 days, ns).

NK3R antagonist had no effect on basal LH secretion and did not affect the magnitude of the mid-cycle LH surge (ANOVA ns), the concentration of which was comparable to the control cycle (31.8±7.8 with NK3R antagonist vs 24.0±4.4 IU/l on LH surge day 0, ns) (Fig. 6.2E). Pre-ovulatory estradiol levels were not different in the NK3R antagonist-treated cycles compared to controls (470±89 with NK3R antagonist vs 690±109 pmol/l on LH surge day -2, ns) and no changes were observed in FSH secretion (Fig. 6.2 F and G). Luteal function was also unaffected by the NK3R antagonist (ANOVA ns) with equivalent mid-luteal progesterone concentrations (NK3R antagonist 37.2±6.7 vs 28.0±2.8 nmol/l day 8-10 of the LH surge, ns) (Fig. 6.2H).

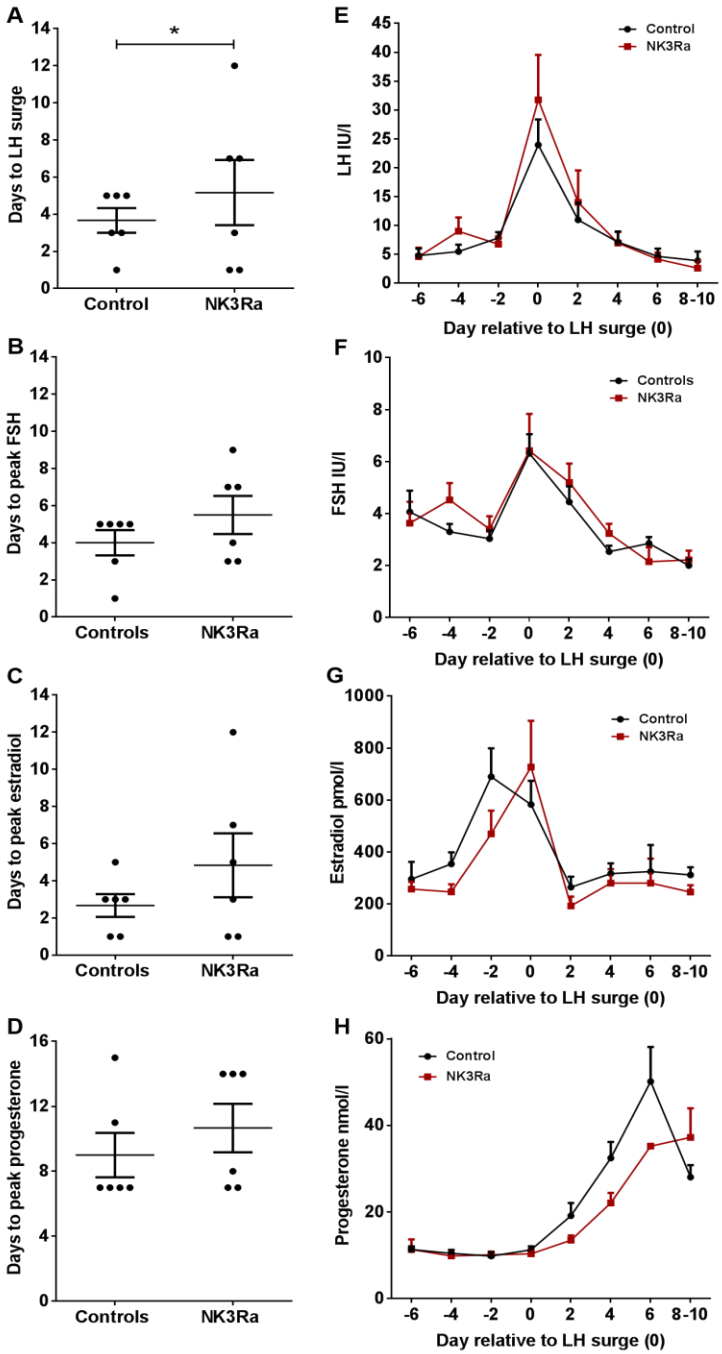


Figure 6.2 Timing of peak reproductive hormone secretion and mean hormone concentrations in the late follicular phase in healthy women with and without NK3R antagonist treatment.

Variation in the number of days from the emergence of a dominant ≥12mm ovarian follicle until the LH surge **(A)** and peak FSH **(B)**, estradiol **(C)** and progesterone **(D)** secretion was tested by Levene's test for homogeneity of variance. Mean reproductive hormone concentrations relative to the LH surge day (0) **(E-H)** in the control and NK3R antagonist-treated women in the late follicular phase were compared by unpaired two-way ANOVA with Bonferroni's multiple comparisons post hoc analysis. Data are presented as mean ± SEM. *, $p<0.05$ Levene's test.

6.4.1.3 NK3R antagonist effects on follicle development and the endometrial response

Individual subject data suggest some variation in the development of a dominant follicle, which was delayed in 3 women with the NK3R antagonist treatment (Fig. 6.3A). The rate of follicle growth appeared smaller in the NK3R antagonist compared to the control group but this was not statistically different (ANOVA ns) (Fig. 6.3B) with the diameter of the pre-ovulatory follicle being comparable to controls (16.5±0.3 vs 18.7±0.8 mm, ns). Endometrial development was unaffected by the NK3R antagonist (Fig. 6.3C).

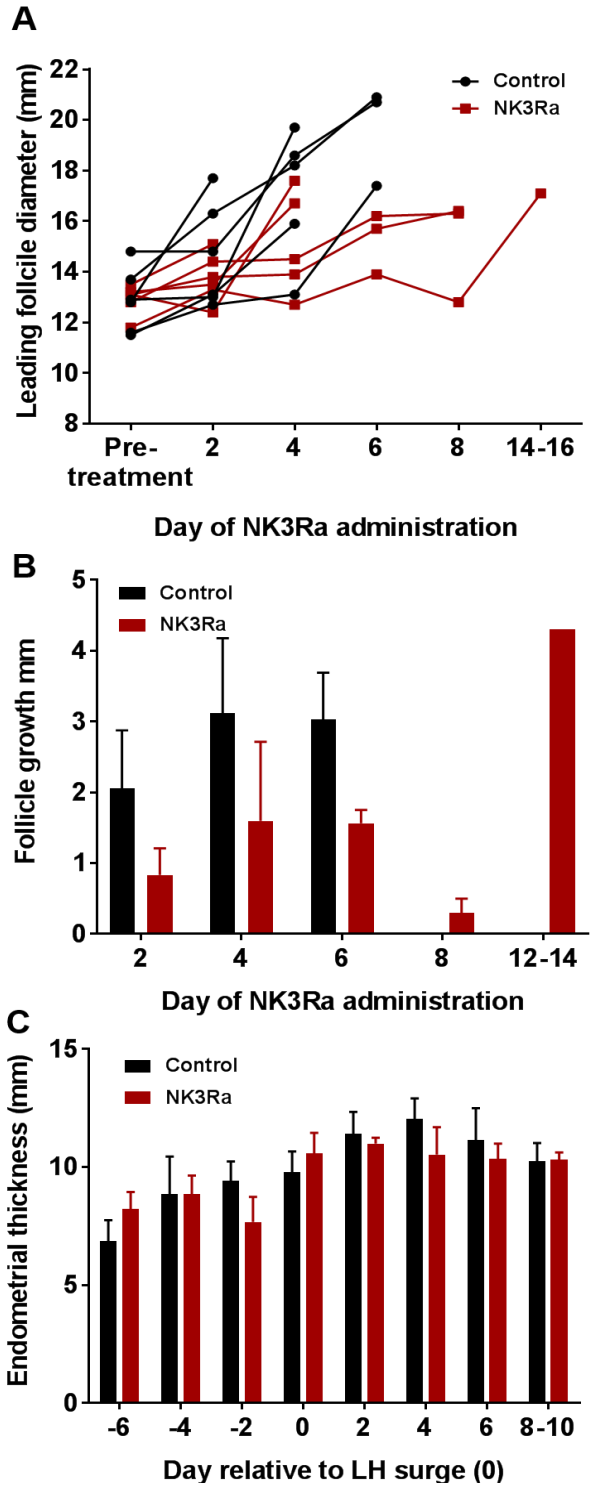


Figure 6.3 NK3R antagonist effects on the development of a dominant ≥12 mm follicle and the endometrial thickness in healthy women in the late follicular phase of menstrual cycle.

(A) Follicle growth in each of the 6 premenopausal women with the NK3R antagonist treatment (*red squares*) compared to the control cycle (black circles). **(B)** Growth in follicle diameter until ovulation and **(C)** endometrial thickness relative to the LH surge day (day 0). Mean ± SEM.

6.4.2 Study 2: Effects of NK3R antagonist on gonadotropin secretion in the luteal phase and luteolysis

6.4.2.1 Baseline characteristics

Each participant took part in control and NK3R antagonist cycles, which were comparable by cycle day on which the study visits commenced and by pre-treatment reproductive hormone profile (Table 6.2).

Characteristic	Control cycle	NK3Ra cycle	p value
Days since ovulation (n)	2.7 ± 0.5	2.5 ± 0.2	0.99
LH (IU)	9.7 ± 1.7	8.2 ± 1.4	0.52
FSH (IU/l)	3.6 ± 0.7	3.8 ± 0.5	0.79
Estradiol (pmol/l)	271 ± 44	306 ± 40	0.44
Progesterone (nmol/l)	22.3 ± 4.8	19.6 ± 4.7	0.99

Table 6.2 Basic characteristics of the participants (n=6) in the luteal phase of menstrual cycle. Subjects in the control and NK3R antagonist-treated cycles were comparable at pre-treatment based on the number of days since ovulation to the start of the at that time. Data are presented as mean ± SEM.

6.4.2.2 NK3R antagonist effects on reproductive hormone secretion

Luteal LH and FSH concentrations were unchanged with the NK3R antagonist (Fig. 6.4 A and B). Although estradiol secretion appeared suppressed by the NK3R antagonist compared to control cycle, this was not significant statistically (ANOVA, ns) (Fig. 6.4C). Luteal function was overall unaffected by the NK3R antagonist with no changes observed in peak estradiol (418±41 vs 569±98 pmol/l, ns) and progesterone (35.9±3.8 vs 45.9± 5.6 nmol/l, ns) secretion between the control and treatment cycles (Fig. 6.4 D-F).

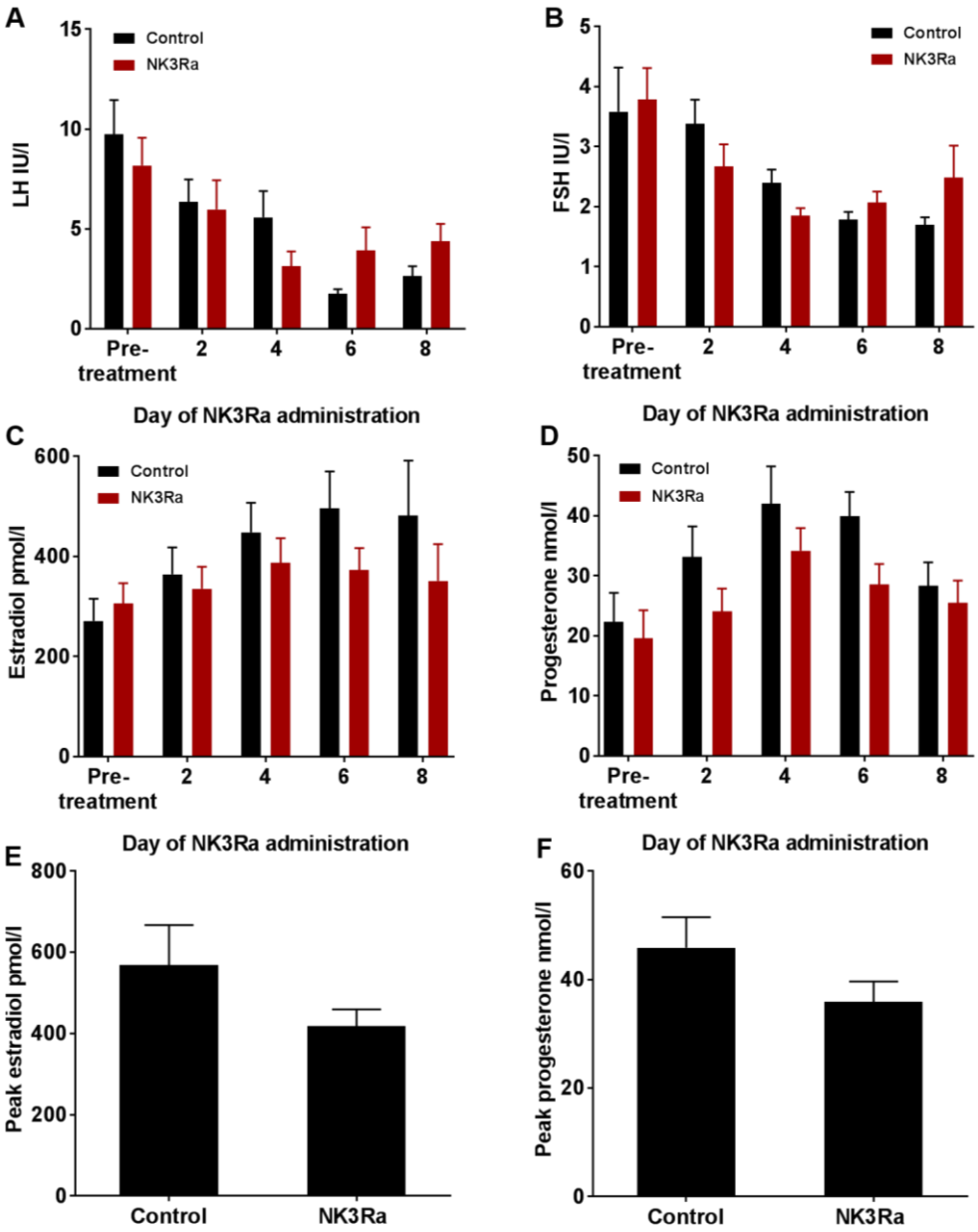


Figure 6.4 Mean luteal reproductive hormone secretion with and without NK3R antagonist administration in women.

Mean LH **(A)**, FSH **(B)**, estradiol **(C)** and progesterone **(D)** secretion and peak estradiol **(E)** and progesterone **(F)** levels in the luteal phase in healthy pre-menopausal women (n=6). Data are presented as mean ± SEM.

NK3R antagonist administration post ovulation in healthy women tightened the variation in the timing of peak estradiol secretion (Levene's test p=0.03) (Fig. 6.5A). No changes were observed in timing of peak progesterone secretion, and the duration of luteal phase was overall unaffected by treatment (Fig. 6.5 B and C).

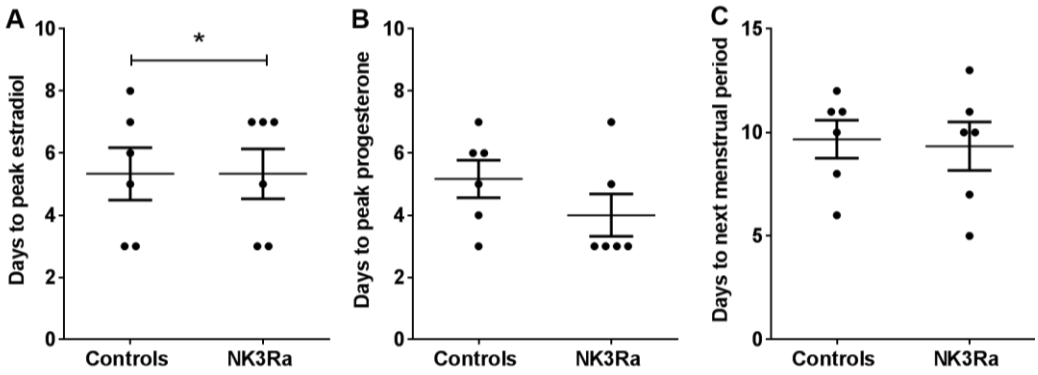


Figure 6.5 NK3R antagonist effects on the timing of luteal hormone secretion and cycle length. Peak estradiol **(A)** and progesterone **(B)** secretion and days to the next menstrual period **(C)** in the control and NK3R antagonist cycle (n=6). Days are counted from the start of the study visits i.e. from day 2-3 of ovulation. Data are presented as mean ± SEM. *, p<0.05 Levene's test.

6.4.2.3 NK3R antagonist effects on the endometrial thickness

NK3R antagonist had no effect on the endometrial thickness in the secretory phase (Fig. 6.6).

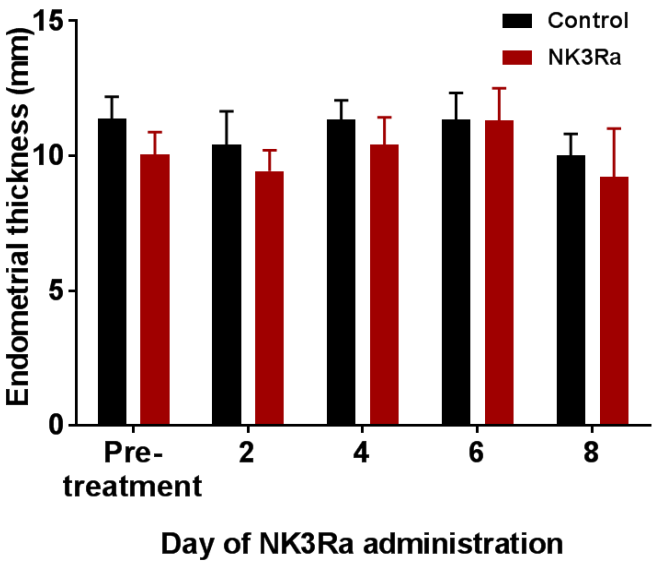


Figure 6.6 Effects of NK3R antagonist on the endometrial response in the luteal phase in healthy premenopausal women.

6.4.3 Tolerability and safety

NK3R antagonist treatment was well tolerated with no treatment discontinuations. Haematology and biochemistry safety parameters remained stable in all subjects throughout the study period.

6.5 Discussion

In this study, the effects of NK3R antagonism in the late follicular and luteal phases were explored in healthy women. Administration of this and similar drugs at these stages of the cycle has not been reported previously. In the late follicular phase, NK3R antagonist administration for 7 days increased the variation in the timing of the LH surge but did not abolish it, suggesting that the timing of positive estrogen feedback to the hypothalamic-pituitary centres was disrupted. While no clear changes were observed in estradiol secretion that triggers the LH surge, the timing of estradiol rise appeared more variable during treatment. However, NK3R antagonist had no marked effect on the growth of an already selected dominant follicle and luteal function, as reflected by progesterone secretion, was also unaffected.

When administered in the luteal phase, NK3R antagonist reduced the variation in the timing of peak estradiol secretion, concentrations of which were also slightly reduced, but it had no overall effect on luteal function and its duration. Although no changes were seen in LH secretion, this may not be detected by performing single timepoint blood sampling, and pulsatile LH secretion was not assessed in this study. Thus it remains possible that NKB is involved in regulating LH secretion in the luteal phase, as LH is the major determinant of androgen availability for estradiol synthesis throughout the menstrual cycle in premenopausal women (Karnitis et al. 1994, Ben-Chetrit et al. 1996).

The present data demonstrate a striking difference in NK3R antagonism effects on the timing of key events in the female reproduction when administered in the late compared to early follicular phase in normal women in Chapter 5. In the early follicular phase, NK3R antagonist delayed ovarian follicle growth and estradiol secretion, the LH surge and ovulation. This resulted in luteal progesterone rise and next menstrual period both being delayed for the duration of 7-day treatment (see

Chapter 5). Similarly, the LH surge was postponed and menstrual cycle length was prolonged in another study administering a different NK3R antagonist (ESN364) in normal women for 21 days throughout the follicular phase (Fraser et al. 2016) with comparable data in female rhesus monkeys (Fraser et al. 2015a). Interestingly, in the late follicular phase NK3R antagonist did not consistently delay the LH surge but perturbed its timing, advancing it in three and delaying it in another three women without any effect on overall reproductive hormone concentrations. Taken together this suggests that in the early follicular phase NKB modulates basal GnRH/LH secretion (as demonstrated by pulsatility studies), but in the late follicular phase NKB may have a role in mediating estrogen feedback and thus the timing of the LH surge without regulating the surge mode of LH secretion. Consistent with this, some variation was seen in the timing of pre-ovulatory estradiol rise but no effect was observed on peak estradiol secretion and LH levels on the surge day. Additional data supporting a key role of NKB signalling in sensing and mediating estrogen feedback are presented in Chapter 7. One explanation for lack of the NK3R antagonist effect on the LH surge levels may be the pituitary response to estradiol as seen in patients with deficient hypothalamic function, such as Kallmann syndrome, during GnRH administration by pump who can show a mid-cycle LH surge without any change in GnRH dose (Crowley and McArthur. 1980). In keeping with our findings, central administration of another NK3R antagonist (MRK-08) in ovariectomised ewes also interfered with the timing of estrogen induced LH surge, delaying the onset-to-peak time of LH secretion but not abolishing it (Li et al. 2015). The anatomical location of NKB neurones within the infundibular nucleus in humans and the equivalent arcuate nucleus in ruminants, the site for positive estrogen feedback in those species, further supports the involvement of this neuropeptide in mediating sex-steroid feedback (Oakley et al. 2009, Pinilla et al. 2012). This is, however, species specific since NKB is thought not to be involved in the pre-ovulatory LH surge in rodents, based on the location of NKB neurones in the arcuate nucleus only, but not the anteroventral periventricular (AVPV) nucleus, the site for positive estrogen action in those species (Oakley et al. 2009, Pinilla et al. 2012). Despite perturbation of the LH surge timing, NK3R antagonist administration in the late follicular phase did not increase the variation in days to peak luteal progesterone secretion and no difference was

observed in length to the next menstrual period between the control and treatment cycle. It is possible that more variable baseline cycle length and therefore follicular phase between the participants and the small sample size precluded demonstration of subtle changes in the timing of luteal sex-steroid secretion and the onset of next menstruation with NK3R antagonist treatment. Using a model to standardise the onset of LH surge between the participants and even between the different cycles of the same participant, would aid clarification for the role of NKB in mediating positive estrogen feedback and the LH surge in humans, which is further explored in Chapter 7.

It is a novel finding that NK3R antagonism had no effect on the growth of a dominant follicle in the late follicular phase, which is in contrast to clear suppression of antral follicle development seen early in the follicular phase before the selection process has occurred. It is therefore likely that basal GnRH/LH secretion is unaffected by the NK3R antagonist in the late follicular phase as ovarian follicle growth was highly sensitive to such changes in the early follicular phase. Both NKB and NK3R are expressed at the mRNA and protein level in human granulosa cells of the pre-ovulatory follicle obtained after ovarian stimulation during assisted conception treatment (Garcia-Ortega et al. 2014, Garcia-Ortega et al. 2016). NKB and NK3R immunoreactivity changes throughout follicle maturation, being minimal in growing follicles but high in corpora lutea in women (Cejudo Roman et al. 2012). The NKB-NK3R system has also been detected in mouse and rat granulosa cells (Loffler et al. 2004, Candenas et al. 2005). Therefore, the present lack of ovarian effect with the NK3R antagonist administration in the late follicular phase further supports the hypothalamic action of NK3R antagonist in mediating suppression of follicle development seen in the early follicular phase. Consistent with our findings, in cultured human mural granulosa cells from oocyte donors undergoing ovulation stimulation treatment, NKB itself had no effect on calcium influx (Garcia-Ortega et al. 2014). In the presence of a cocktail of antagonists selective for NK1R, NK2R and NK3R, calcium influx was reduced, but this was to a lesser extent with NK3R antagonist alone (Garcia-Ortega et al. 2014). Of note, expression of the NKB-NK3R system in the follicles obtained from IVF cycles may not represent that of a normally

cycling woman and ovarian stimulation can induce changes in serum and follicular fluid and therefore granulosa cell gene and protein expression (de los Santos et al. 2012). Nevertheless, this suggests that NKB may mediate its local ovarian effects by its interaction with different tachykinin receptors, since NKB is known to activate any of the three tachykinin group receptors (Maggi. 2000, Almeida et al. 2004, Satake et al. 2013).

The lack of suppressive effect of the NK3R antagonist on the growth of a dominant follicle in this study indicates that the NKB-NK3R pathway has limited activity at this stage of the menstrual cycle, although only one dose level was investigated and the limited effects seen here may be greater if higher doses are used. In keeping with this, estrogen exposure decreases *TAC3* and *TACR3* expression in various tissues, including the hypothalamus and uterus (Rance and Young. 1991, Pinto et al. 1999, Pinto et al. 2009, Rance. 2009), suggesting that high estrogen levels in the follicular phase might have donwregulated hypothalamic NK3R expression, the substrate for the AZD4901 antagonist in this study.

The present study has also investigated effects of the blockade of NKB-NK3R pathway on luteal function when administered in both the late follicular and luteal phases of the menstrual cycle. When administered in the late follicular phase, NK3R antagonist neither affected the time to peak luteal progesterone secretion, nor its mid-luteal concentration, suggesting that luteal function and its duration was unaffected by treatment. A different NK3R antagonist (ESN364) attenuated the mid-luteal progesterone rise in rhesus female monkeys, but treatment was initiated in the early follicular phase for 35 days (Fraser et al. 2015a). Similarly, delayed luteal progesterone secretion was observed in healthy women with 21-day administration of NK3R antagonist (ESN364) administration (Fraser et al. 2016). Changes seen in luteal sex-steroid secretion in previous human and animal studies therefore reflect effects of NK3R antagonism on hormone release earlier in the cycle, but do not determine the role of NKB on luteal function. This study demonstrates that when administered 2-3 days after ovulation, NK3R antagonist decreased the variation in days to peak estradiol secretion and appeared to slightly suppress luteal estradiol release, but these effects were modest. This may indicate a locus of action of the

NK3R antagonist at the ovarian level in this experimental design, as there is abundant expression of NKB-NK3R in the corpus luteum (Cejudo Roman et al. 2012, Garcia-Ortega et al. 2014, Garcia-Ortega et al. 2016), although GnRH/LH pulsatility assessment was not performed here and a dual site of action therefore cannot be ruled out. Peak luteal estradiol and progesterone secretion was unaffected by the treatment and the duration of luteal phase was unchanged, suggesting overall normal luteal function in the presence of NK3R antagonist.

Local uterine effects were not explored in this study, but this is unlikely since no effect was observed on luteal hormone secretion and endometrial thickness with this dose of NK3R antagonist in the late follicular and luteal phases in healthy women. Nevertheless, the NKB-NK3R pathway is localised to the mammalian uterus (Pinto et al. 1999, Patak et al. 2003, Pinto et al. 2009, Cejudo Roman et al. 2012) and its expression is dependent on the hormonal state. In the human myometrium the immunohistochemical expression of NK3R was weaker in the proliferative compared to the secretory phase (Cejudo Roman et al. 2012), consistent with reduced *TACR3* expression in the human and rodents uteruses with estrogen dominance (Pinto et al. 1999, Patak et al. 2003, Pinto et al. 2009). The presence of NKB and NK3R in uterine mast cells and vascular smooth muscle suggests that the system may be a link between the uterus and immune system, the involvement of which has been increasingly recognised in heavy menstrual bleeding (Maybin et al. 2011a, Maybin et al. 2011b). Furthermore, the expression of *TAC3* and *TACR3* was significantly upregulated and their cyclical variation was reduced in uterine fibroids following hysterectomy, suggesting that dysregulation of NKB-NK3R signalling might contribute to symptoms seen in women with fibroids (Canete et al. 2013). In rhesus monkeys, endometrial assessment showed moderate stromal atrophy and mucosal glandular inactivity with NK3R antagonist treatment throughout the menstrual cycle, the effects of which were reserved in the next menstrual cycle upon treatment cessation (Fraser et al. 2015a). Histological analysis of endometrium exposed to NK3R antagonist has not yet been performed in humans. Manipulation of NKB-NK3R system clearly opens new paradigms for therapeutic application in the regulation of female reproduction, including birth control and management of sex-

steroid dependent disorders. Nevertheless, there remains a necessity to ensure normal endometrial function in women of reproductive age post NK3R antagonist exposure before translational application of NK3R blockade.

In summary, NK3R antagonism in the late follicular phase in healthy premenopausal women altered the timing of the LH surge but there were no detected effects on pre-ovulatory LH secretion, or late follicle growth and estradiol production. This suggests the involvement of NKB in positive estrogen feedback and the timing of the mid-cycle LH secretion but a more limited role in basal LH secretion in the late follicular phase (although pulsatility analysis was not performed). This is in contrast to the early follicular phase, where basal GnRH/LH secretion was reduced and ovarian follicle development was suppressed. When administered in the luteal phase, this dose and course of NK3R antagonist administration affected neither luteal function, nor its duration. Taken together, these data confirm the involvement of NKB in physiological regulation of female reproduction in the normal dynamics of the menstrual cycle but the extent of which is dependent on the phase of the cycle, possibly reflecting changes in the sex-steroid environment. Our studies add further understanding to the neuroendocrine control of key events in human fertility.

Chapter 7. Interactions between neurokinin B and kisspeptin in mediating estrogen feedback in healthy women

The work in this chapter has been published in the Journal of Clinical Endocrinology and Metabolism:

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Acknowledgements and author contributions for the above manuscript are detailed in Chapter 1.

7.1 Introduction

In Chapters 5 and 6, the role of NKB in regulating gonadotropin secretion and ovarian function and the influence thereon of varying sex-steroid milieu across the menstrual cycle in healthy women was investigated. In the early follicular phase, NKB action on LH secretion was identified, and found to be by modulation of basal GnRH release, which was decreased by the NK3R antagonist also suppressing ovarian estrogen secretion and delaying LH surge and ovulation. When administered in the late follicular phase, NK3R antagonist interfered with the timing of LH surge but did not inhibit pre-ovulatory estradiol or LH secretion. Similarly, LH surges were observed in all ovariectomised estrogen challenged sheep following an administration of a different NK3R antagonist (MRK-08), but the time from LH surge onset to peak was significantly delayed (Li et al. 2015). This suggests differential action of NKB in regulating downstream GnRH/LH secretion across the menstrual cycle, which might be dependent on sex-steroid feedback. Although NKB controls basal GnRH/LH secretion early in the cycle when estrogen feedback is negative and estradiol levels are relatively low, the role of NKB at the switch to positive estrogen feedback remains unclear. We observed no changes in LH secretion with the NK3R antagonist in the late follicular phase in healthy women, but LH pulsatility assessment was not performed and subtle effects on LH release may have been overlooked. Furthermore, timing of the physiological LH surge was variable

between the participants, introducing difficulty in accurate assessment. We have therefore used a model of exogenous estradiol (transdermal) administration developed by Baird *et al*., (Baird et al. 1995) standardising estrogen exposure and the onset of increased LH secretion to further explore the role of NKB in regulating sex-steroid feedback at the time of the switch from negative to positive, and pulsatile LH secretion during this feedback in healthy women.

NKB neurones in the infundibular nucleus in humans, equivalent to the arcuate nucleus in rodents and other species, also co-express kisspeptin, and the functions of those neuropeptides are closely interlinked (Pinilla et al. 2012). NKB and kisspeptin are essential for normal GnRH and gonadotropin secretion as inactivating mutations in NKB, kisspeptin and their receptors are associated with hypogonadotropic pubertal delay (de Roux et al. 2003, Seminara et al. 2003, Topaloglu et al. 2009, Topaloglu et al. 2012), whilst activating mutations in kisspeptin-kisspeptin receptor pathway cause precocious puberty (Teles et al. 2008). Experimental characterisation of the relative roles played by kisspeptin and NKB, as well as their functional hierarchy, has been largely carried out in non-human models (Billings et al. 2010, Corander et al. 2010, Navarro et al. 2011a, Ramaswamy et al. 2011, Garcia-Galiano et al. 2012). The first human study demonstrating the interaction between kisspeptin and NKB was in a disease model, where in patients with loss of function mutations in NKB and its receptor, kisspeptin-10 infusion increased LH secretion and restored its pulsatility (Young et al. 2013). This, and concordant data from animal models (Corander et al. 2010, Garcia-Galiano et al. 2012), has led to the conclusion that central NKB signalling is functionally upstream of kisspeptin. However, there are no equivalent data on this in humans in health. We have shown that in states of slow LH output, NK3R antagonist suppressed LH and testosterone secretion in men by modulating nonpulsatile GnRH secretion, but did not abrogate stimulatory kisspeptin-10 response (Chapter 3). In postmenopausal women with high LH output, gonadotropin response to kisspeptin-10 and pharmacological blockade of NK3R was rather limited (Chapter 4). Taken together our data support the overall hierarchy whereby NKB is functionally upstream of kisspeptin, but suggest that this interaction is complex and may be influenced by the sex-steroid milieu. Indeed, in another

study, the stimulatory effect of kisspeptin-54 on LH and testosterone secretion was greater than that observed with co-infusion of NKB itself and kisspeptin-54 (Narayanaswamy et al. 2015). LH response to exogenous kisspeptin is greatest in the late follicular phase (Dhillo et al. 2007, Jayasena et al. 2011, Chan et al. 2012) and is positively related to endogenous estradiol levels (Narayanaswamy et al. 2015), implicating kisspeptin in mediating positive sex-steroid feedback on LH release. However, there are no studies exploring the interaction between kisspeptin and NKB at this stage of the menstrual cycle. The antagonistic effect of neurokinin B and its interaction with kisspeptin was explored during exogenous estrogen administration, aiming to reveal the functional hierarchy of kisspeptin and NKB at the time of positive estrogen feedback and the LH surge in women.

7.2 Objectives

The objectives of the study were, using a model of estrogen-induced LH secretion in healthy premenopausal women:

1) To determine the effect of KP-10 on gonadotropin secretion;
2) To determine the effect of NK3R antagonist on gonadotropin secretion;
3) To investigate the effect of NK3R antagonist on LH pulsatility;
4) To determine if KP-10 stimulated LH secretion is abrogated in the presence of NK3R antagonist, thereby revealing the functional hierarchy of neurokinin B being proximal to kisspeptin.

7.3 Materials and Methods

7.3.1 Participants

Twenty healthy women, aged 18-45 years with regular menstrual cycles (25-35 days) were recruited from the community to this study, which was approved by South East Scotland Research Ethics Committee (Ref: 09/S1101/67); all volunteers provided informed written consent. Subjects were not taking steroidal contraception, had normal physical examination, and full blood count, renal function, electrolytes, liver function and electrocardiogram were within normal limits.

7.3.2 Study drugs

Kisspeptin-10 was custom synthesized under GMP standards (Bachem GmBH, Weil am Rhein, Germany) (George et al. 2011). 1 mg kisspeptin-10 was dissolved in 5 ml sterile normal (0.9%) saline immediately before infusion. The syringe and line for infusion were first coated for 30 min with kisspeptin-10 to minimise peptide loss from adherence to the plastic. Sterile normal saline was infused as vehicle. The specific neurokinin-3 receptor (NK3R) inhibitor AZD4901, formulated as 20 mg tablets, was gifted by AstraZeneca, UK. Transdermal patches releasing 200 μg 17β estradiol per 24 hours (Janssen-Cilag Buckinghamshire, UK) were used as exogenous estradiol treatment (Medicines.org. 2015).

7.3.3 Protocol

To standardise estrogen exposure and the onset of increased LH secretion, we used a model of follicular phase administration of transdermal estradiol (200μg/day), which initially suppresses then at 48 hours increases LH secretion (Baird et al. 1995). We confirmed that LH secretion at 48 hours is increased to the same extent if the patches were removed at 32 hours or continued till 72 hours: for the main study therefore patches were removed at 32 hours (Fig. 7.1). Sample size was based on previous proof of concept mechanistic studies (George et al. 2011, George et al. 2012).

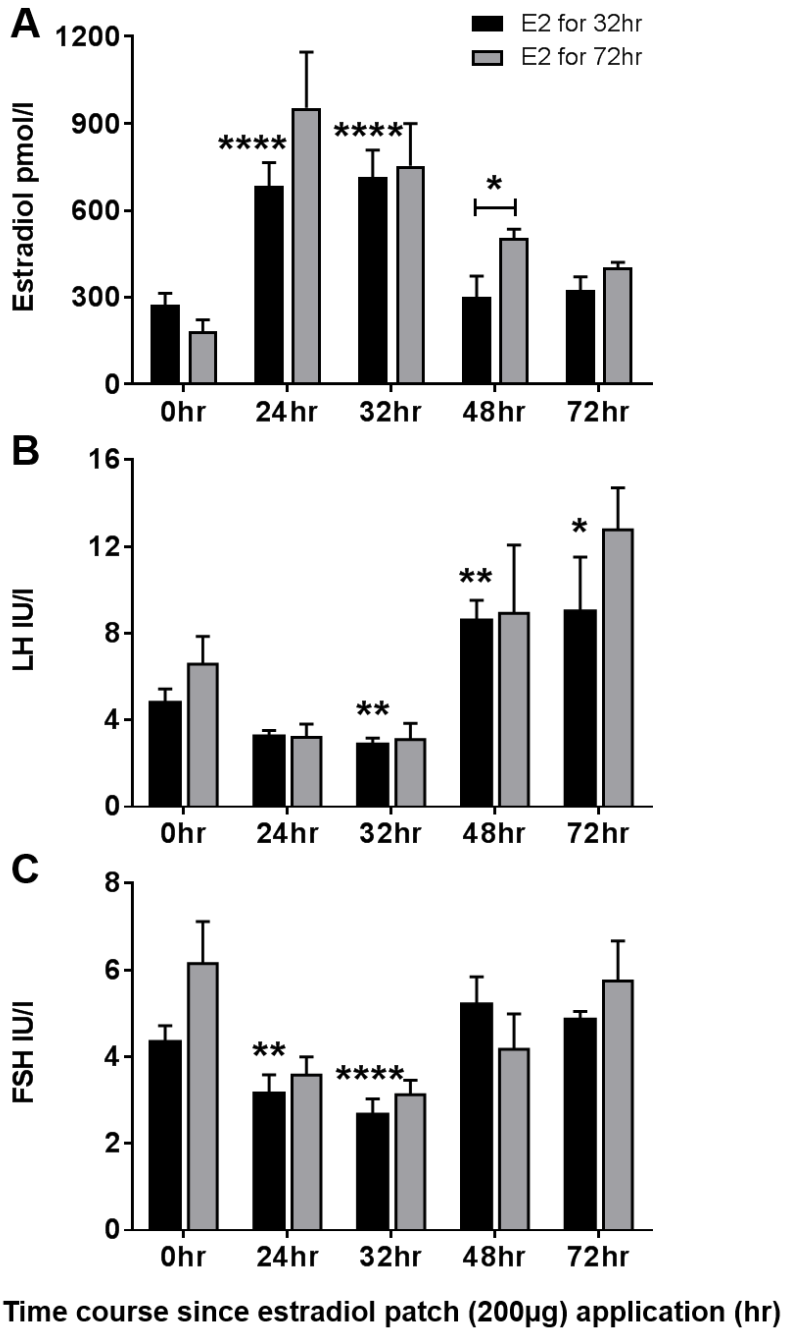

Figure 7.1 Model of estrogen-induced LH secretion.

Estradiol **(A)**, LH **(B)** and FSH **(C)** levels in a model of estrogen-induced LH secretion at 48 hours with ten women receiving estrogen for 32 hr (black bars) and four women receiving estrogen treatment for 72 hr (grey bars). No kisspeptin-10 or NK3R antagonist was administered. Two estradiol patches releasing a total of 200 µg estradiol/day were applied at 0 hours. Gonadotropin levels were equivalent between estrogen exposure for 32 and 72 hours. Data presented as mean ± SEM. *, $p<0.05$; **, $p<0.01$; ****, $p<0.0001$ vs 0 hours, except where a difference between groups is indicated in estradiol concentrations.

Twenty women were randomly allocated to NK3R antagonist (AZD4901) 40mg oral twice daily starting from cycle day 4-6 for 6 days, or no treatment (Fig. 7.2). Two transdermal estradiol patches were administered after 5 days (time 0 hours), in the late follicular phase (cycle day 9-11, according to the day of starting AZD4901). At 24 hours of estradiol treatment volunteers attended our clinical research facility for 8 hours. After an hour of baseline sampling, volunteers were randomised (using sealed envelopes) to receive a continuous intravenous infusion of kisspeptin-10 (4µg/kg/hour) or vehicle for 7 hours. In the NK3R antagonist treatment group, the last dose of AZD4901 was on the morning of kisspeptin-10 or vehicle administration. Estradiol patches were removed at the end of the infusion i.e. 32 hours after application. Volunteers attended for further measurement of reproductive hormones at 48 and 72 hours. In a subsequent menstrual cycle, all women returned to receive the alternate infusion of kisspeptin-10 or saline. Those receiving NK3R antagonist had at least one wash out cycle between treatment cycles. To compare the effect of exogenous vs endogenous estrogen on kisspeptin-10 response, another group of ten women received intravenous kisspeptin-10 (4µg/kg/hour) infusion for 7 hours on cycle day 10-12 without exogenous estrogen treatment, with reproductive hormone measurements at equivalent time points.

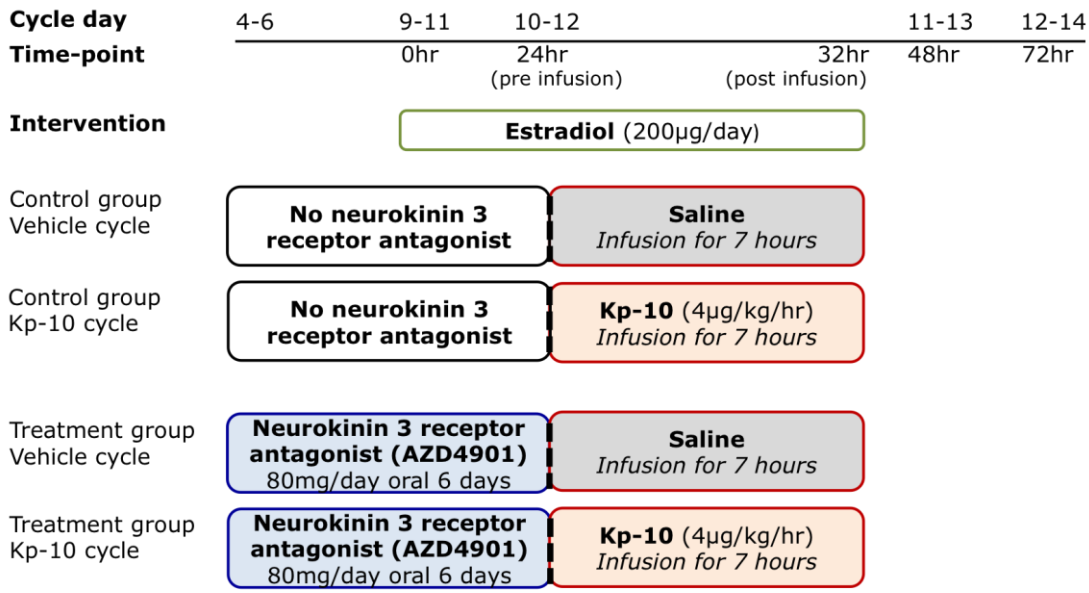

Figure 7.2 Study protocol diagram.

Follicular phase administration of transdermal estradiol was used to induce LH secretion 48 hours later as a model of the mid-cycle LH surge in women. Ten healthy women were administered NK3R antagonist AZD4901 from cycle day 4-6 for 6 days, matched to ten women having no treatment. Transdermal estradiol was applied after 5 days. 24 hours later, women were randomised to 7 hour of kisspeptin-10 or vehicle infusion, returning in a subsequent cycle for the alternate infusion. Reproductive hormones were measured throughout the study and LH pulsatility assessed during 10 minute blood sampling for 8 hours.

7.3.4 Blood sampling and hormone assays

Peripheral venous blood was sampled for LH, FSH and estradiol in the treatment group on the day of commencing NK3R antagonist and in both control and treatment groups before estradiol treatment (0 hour) and then at 24, 32, 48 and 72 hours. During the 8 hour visit, blood samples were collected via an indwelling iv cannula at 10 min intervals for assessments of LH pulsatility; FSH was measured hourly. Blood samples were centrifuged at 4°C for 10 min at 3000 rpm and serum frozen at -20°C or below until analysis. LH and FSH were determined by ELISA as previously described (George et al. 2011). 17β-estradiol was measured by ELISA (Demeditec Diagnostics, Kiel, Germany). Inter-assay and intra-assay coefficient of variation for all hormones was <5% at the concentrations measured. Lower detection limit for LH and FSH was 0.1 IU/l and for estradiol 20pmol/l.

7.3.5 Statistical analysis

Analysis of variance (ANOVA) was used to analyse preliminary data on LH changes with time in the model. For the primary endpoints, hormone concentrations were compared between the four treatment groups at specific time points using ANOVA with repeated measures as appropriate. If there was overall significance, post hoc analysis was performed with Bonferroni's correction for multiple comparisons, comparing all four treatments simultaneously at each time point. The relationship between the timing of peak LH and treatment was assessed by chi-square test. Pearson correlation coefficient was computed to assess the relationship between estradiol concentrations and LH response to kisspeptin-10.

The number of LH pulses, secretory mass of LH per pulse, basal (nonpulsatile) and pulsatile (integral of dual amplitude and frequency regulation) LH secretion were identified by an established deconvolutional algorithm with cluster analysis (93% sensitivity and specificity) (Veldhuis et al. 2008, Liu et al. 2009) blinded to treatment allocation. Approximate entropy (ApEn), a measure of orderliness, was also estimated for the pattern of LH secretion. Deconvolutional estimates and mean hourly hormone changes were not calculated for one woman in each group, as full 8 hour sampling data were not obtained. ANOVA was used to assess changes in LH pulsatility parameters between the 4 groups, with post hoc testing as above.

Data are presented as mean ± SEM. Data not normally distributed were log-transformed prior to statistical analysis, resulting in a distribution that approximated a normal distribution. Differences were regarded as significant at a two-sided $p<0.05$. The statistical software package GraphPad Prism (GraphPad, San Diego, California) was used.

7.4 Results

Baseline age, BMI and the menstrual cycle length were comparable between the subjects in the control and the treatment group, as were baseline LH, FSH and estradiol levels in vehicle and kisspeptin-10 cycles within the group (Table 7.1).

	Control group			**Treatment (NK3Ra) group**		**p value**
n	10			10		
Age (years)	35 ± 1.8			35 ± 1.7		ns
BMI (kg/m^2)	25 ± 1.4			28 ± 2.2		ns
Cycle length (days)	29 ± 0.6			28 ± 0.5		ns
	Vehicle cycle	**Kp-10 cycle**	**p value**	**Vehicle cycle**	**Kp-10 cycle**	**p value**
Menstrual cycle day	9.2 ± 0.2	9.4 ± 0.3	ns	4.4 ± 0.2	4.8 ± 0.2	ns
LH (IU/l)	4.8 ± 0.7	5.6 ± 0.6	ns	5.0 ± 0.6	4.9 ± 0.6	ns
FSH (IU/l)	4.3 ± 0.4	5.3 ± 0.8	ns	6.2 ± 0.6	5.5 ± 0.4	ns
Estradiol (pmol/l)	274 ± 40	287 ± 50	ns	121 ± 17	154 ± 15	ns

Table 7.1 Baseline characteristics of women in the control and the treatment group undergoing vehicle and kisspeptin-10 infusion.

Data are shown as mean ± SEM; ns, not significant. Note that baseline data on control and treatment groups in lower part of the table reflect sampling at different stages of the menstrual cycle.

7.4.1 Model validation for estrogen-induced LH secretion

Treatment with exogenous estrogen for 32 hours increased serum estradiol concentrations as expected ($p<0.0001$) (Fig. 7.1). Serum LH was initially suppressed at 32 hours of estrogen treatment, then increased at 48 hours which persisted at 72 hours (all $p<0.05$ vs 0 hours). FSH concentrations were significantly lower at 24 ($p<0.01$) and 32 hours ($p<0.0001$) but were not higher at 48 and 72 hours compared to baseline. This confirms that with this regimen, estrogenic negative feedback is followed by increased LH secretion, thus standardising estrogen exposure and the time course of changes in LH secretion.

7.4.2 Kisspeptin-10 stimulates gonadotropin secretion

During estrogen administration, kisspeptin-10 stimulated LH secretion to 16.4±3.9 IU/l at the end of infusion vs 2.9±0.3 IU/l following vehicle administration ($p<0.0001$) (Fig. 7.3A). The time course of this response is shown in Fig 7.4A. Kisspeptin-10 induced LH secretion persisted beyond the discontinuation of the infusion with higher peak LH compared to controls at 48 hours (9.3±0.7 vs 21.6±5.8 IU/l, $p=0.007$) (Table 7.2). Clarification of the impact of exogenous estradiol on this response was demonstrated in a separate group of women receiving kisspeptin-10 infusion only in the late follicular phase without exogenous estrogen administration, who showed a similar acute increase in LH secretion correlating positively with estradiol concentration ($r^2=0.63$, $p=0.006$), but of a shorter duration (48 hours: 6.8±1.8 IU/l vs 15.0±3.6 with estrogen treatment, $p<0.01$; Fig. 7.5). All subjects in the endogenous estrogen group had peak LH at the end of kisspeptin-10 infusion, whereas in exogenous estrogen-treated subjects the kisspeptin-10 induced peak LH persisted beyond kisspeptin-10 infusion with 50% of women having peak LH at 32 hours and 50% at 48 hours ($p<0.01$, Table 7.2).

FSH secretion was also significantly higher at the end of kisspeptin-10 infusion compared to vehicle in the control group ($p<0.05$) but not different to baseline (0 hours; Fig. 7.3B, 7.4B). As expected with this model of exogenous estradiol administration, estradiol concentrations were similar in kisspeptin-10 and vehicle-infused controls (Fig. 7.3C).

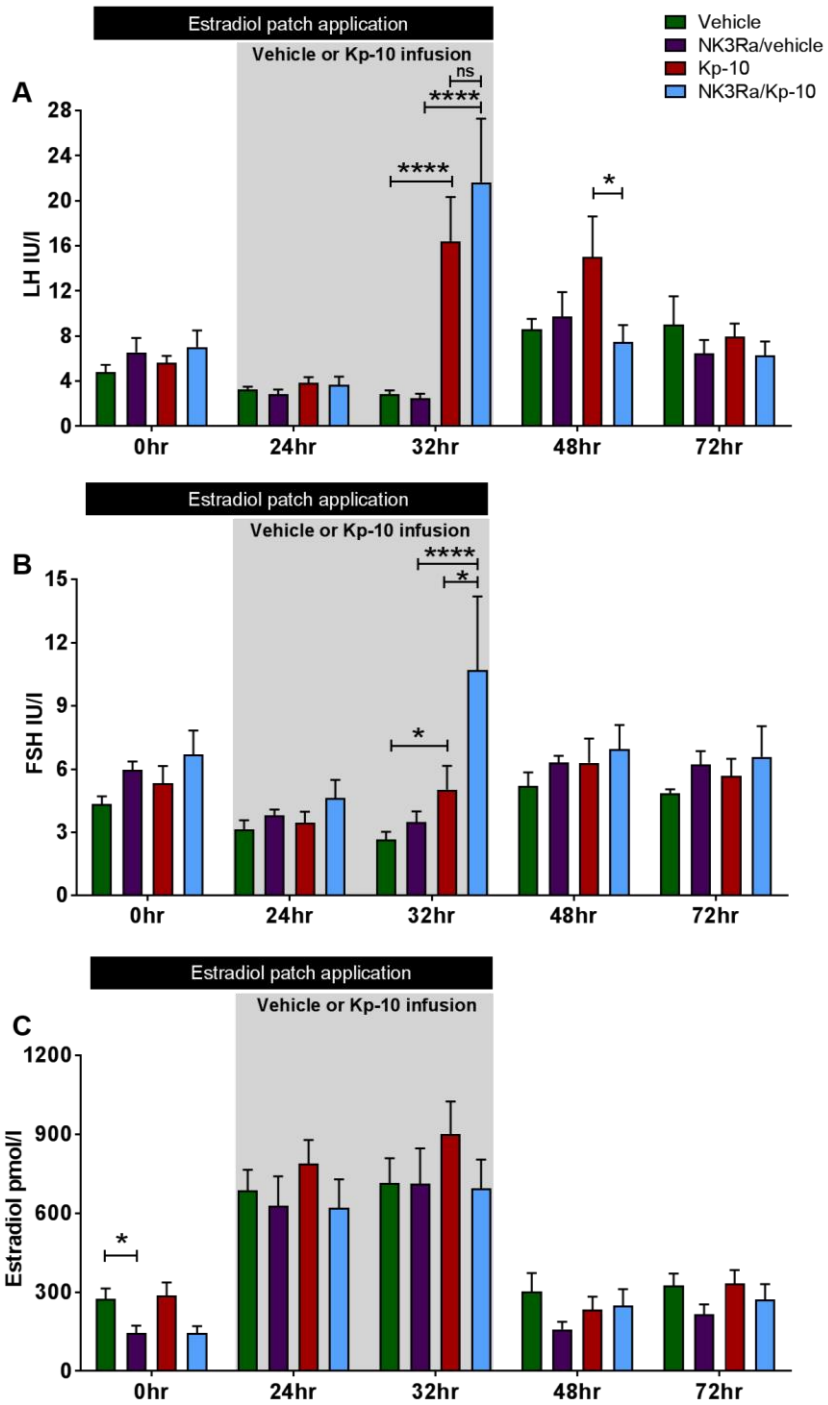

Figure 7.3 Reproductive hormone response to kisspeptin-10 and NK3R antagonist administration in the model of estrogen-induced LH secretion.

Comparison of mean LH **(A)**, FSH **(B)** and estradiol **(C)** response to an infusion of kisspeptin-10 and vehicle in ten control and ten NK3R antagonist-treated cycles in the model of estrogen-induced LH secretion. Two estradiol patches releasing a total of 200 µg estradiol/day were applied between 0 and 32 hours. The infusion period of kisspeptin-10 and vehicle was between 24 and 32 hours. Data presented as mean ± SEM. *, $p<0.05$; ****, $p<0.0001$.

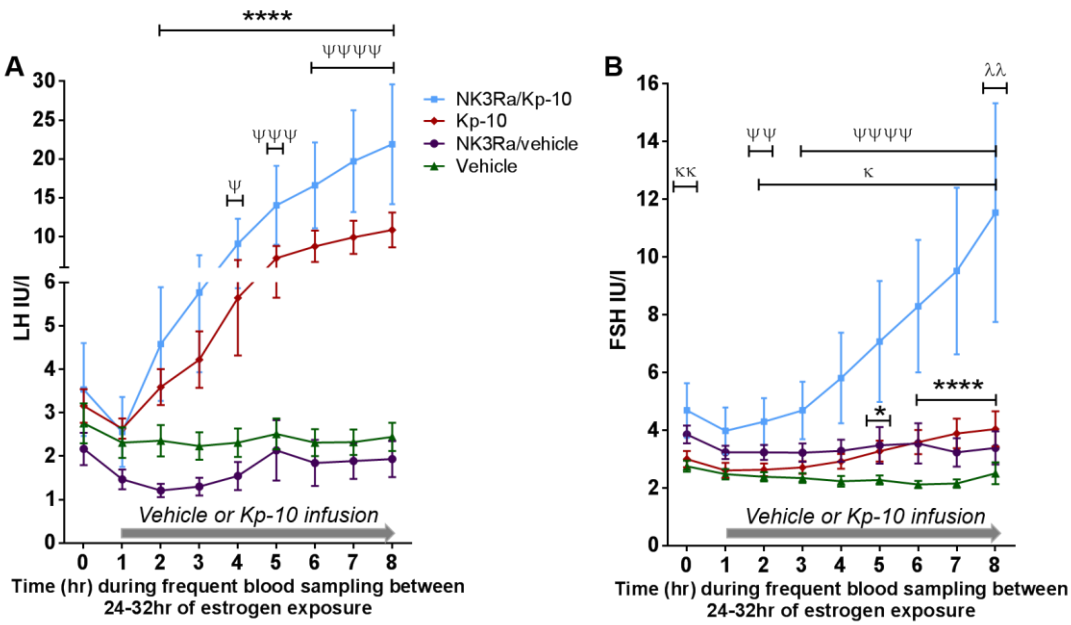

Figure 7.4 Time course analysis of reproductive hormone secretion during 8 hours of frequent blood sampling during vehicle or kisspeptin-10 infusion with and without the NK3R antagonist. LH **(A)** and FSH **(B)** levels an hour pre and during 7 hours of vehicle or kisspeptin-10 infusion in the control group (n=9) and in the treatment (NK3R antagonist) group (n=9). The infusion period of kisspeptin-10 and vehicle was between 24 and 32 hours of estrogen administration. Data presented as mean ± SEM. Statistical analysis by 2-way ANOVA determined statistically lower LH levels between vehicle and NK3Ra-treated women ($p<0.0001$), although Bonferroni's post hoc multiple comparison test found no significant changes at specific time points. For vehicle vs kisspeptin-10 infused controls: $^{*}p<0.05$, $^{****}p<0.0001$. For kisspeptin-10 infusion in controls vs NK3Ra: $^{\lambda\lambda}p<0.01$. For vehicle vs NK3Ra: $^{\kappa}p<0.05$, $^{\kappa\kappa}p<0.01$. For vehicle vs kisspeptin-10 in NK3Ra-treated women: $^{\Psi}p<0.05$, $^{\Psi\Psi}p<0.01$, $^{\Psi\Psi\Psi}p<0.001$, $^{\Psi\Psi\Psi\Psi}p<0.0001$.

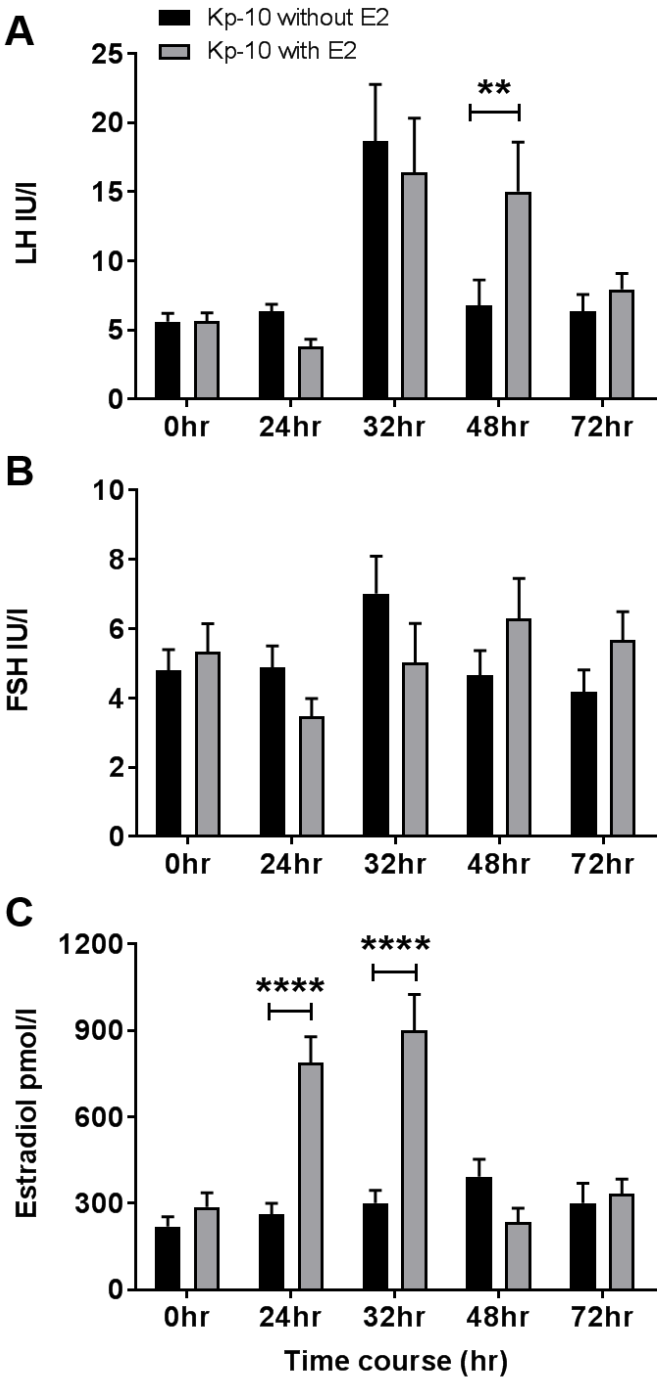

Figure 7.5 The effect of endogenous compared with exogenous estrogen on response to kisspeptin-10 infusion.

LH **(A)**, FSH **(B)** and estradiol **(C)** levels in women receiving kisspeptin-10 infusion (between 24 and 32 hours) without (black bars, n=10) and with (grey bars, n=10) transdermal estradiol treatment. Two estradiol patches releasing a total of 200 μg estradiol/day were applied between 0 and 32 hours. Data presented as mean ± SEM. **, $p<0.01$; ****, $p<0.0001$.

		Timing and mean of peak LH and FSH			
Group	**Test**	**32 hr**	**48 hr**	**72 hr**	**P value**
Vehicle	n peak LH	0 (0%)	7 (70%)	3 (30%)	a*
	LH IU/l		9.3±0.7 a**	15.4±7.7	
	n peak FSH	0 (0%)	5 (50%)	5 (50%)	a^{ns}
	FSH IU/l		6.5±0.7 a^{ns}	5.0±0.3 a^{ns}	
No E2 Kp-10	n peak LH	10 (100%)	0 (0%)	0 (0%)	b**
	LH IU/l	18.7±4.1 b^{ns}			
	n peak FSH	10 (100%)	0 (0%)	0 (0%)	b*
	FSH IU/l	7.0±1.1 b^{ns}			
Kp-10	n peak LH	5 (50%)	5 (50%)	0 (0%)	
	LH IU/l	15.7±4.1	21.6±5.8		
	n peak FSH	3 (30%)	3 (30%)	4 (40%)	
	FSH IU/l	5.2±1.7	10.1±2.7	5.5±0.4	
NK3Ra and vehicle	n peak LH	0 (0%)	9 (90%)	1 (10%)	c***
	LH IU/l		10.1±2.4	6.2	
	n peak FSH	0 (0%)	8 (80%)	2 (20%)	c*
	FSH IU/l		6.2±0.4 c^{ns}	9.6±1.4	
NK3Ra and Kp-10	n peak LH	9 (90%)	1 (10%)	0 (0%)	d^{ns}
	LH IU/l	23.3±6.0 d^{ns}	12.0		
	n peak FSH	6 (60%)	3 (30%)	1 (10%)	d^{ns}
	FSH IU/l	14.9±5.2 d^{ns}	6.5±0.9 d^{ns}	4.5	

Table 7.2 The timing and peak LH and FSH concentration in kisspeptin-10 and vehicle infused subjects in control and treatment (NK3Ra) group.

Numbers in parentheses indicate row percentages. Peak LH and FSH was determined for each participant during 32, 48 or 72 hour time points and mean ± SEM calculated a: vehicle vs kisspeptin-10); b: kisspeptin-10 with and without exogenous estrogen); c: vehicle vs kisspeptin-10 in NK3Ra-treated group); d: kisspeptin-10 with and without NK3Ra). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

7.4.3 NK3R antagonist has differential effects on LH and FSH secretion

Serum LH levels did not change after 5 days of NK3R antagonist treatment (before the estradiol patches were applied) when compared to either pre-treatment concentrations (pre NK3Ra 5.0±0.6 vs 5 days NK3Ra 6.6±1.3 IU/l, ns) or to controls (Fig. 7.3A). Overall there was no difference in LH concentrations and the timing of peak LH in controls vs NK3Ra-treated women (Table 7.2 and Fig. 7.3A). To detect subtle changes in hormone secretion potentially overlooked by single time point blood sampling, analysis of hourly LH for 8 hours post dose showed that overall LH secretion was lower in NK3Ra-treated women compared to controls ($p<0.0001$, Fig. 7.4A), although post hoc analysis indicated no significant differences in LH levels at any individual hourly time point.

FSH concentrations appeared higher throughout treatment with NK3R antagonist compared to controls (Fig. 7.3B) and were significantly higher in NK3Ra-treated women throughout the eight-hour period (i.e. during saline infusion, $p<0.0001$) (Fig. 7.4B). This may reflect that serum estradiol concentrations were significantly lower after 5 days of treatment with NK3R antagonist compared to controls ($p<0.05$) (Fig. 7.3C) and comparable to estradiol levels before NK3R antagonist administration (pre NK3Ra: 121±17 vs 145±27 pmol/l after 5 days NK3Ra, ns).

7.4.4 Effect of NK3R antagonist on the gonadotropin response to kisspeptin-10

NK3R antagonist non-significantly increased kisspeptin-10 stimulated LH secretion at 32 hours (21.6±5.6 with NKB antagonist vs 16.4±3.9 IU/l kisspeptin-10 alone, $p=0.41$) (Fig. 7.3A, 7.4A). The FSH response to kisspeptin-10 was however significantly more pronounced in the presence of NK3Ra (10.7±3.5 vs 5.0±1.1 IU/l at 32h, $p<0.05$ Fig. 7.3B; and throughout the 7 hour infusion: $p<0.0001$ Fig. 7.4B).

However, NK3Ra blunted the duration of kisspeptin-10 induced LH secretion, with significantly lower LH at 48 hours (15.0±3.6 vs 7.5±1.5 IU/l, $p<0.05$) when compared to kisspeptin-10 infused controls, whereas FSH showed no significant difference (Fig. 7.3). There were related changes in the timing of the LH peak (although not statistically significant), which was at 32 hours in 9/10 NK3Ra-treated

women in response to kisspeptin-10 infusion, compared with kisspeptin-10 treated controls whose LH peak timing was evenly divided between 32 and 48 hours (Table 7.2).

7.4.5 NK3R antagonist impedes estradiol dependent kisspeptin-10 response

The relationship between LH response to kisspeptin-10 and estradiol exposure, and the influence thereon of NK3Ra treatment, was investigated by analysing LH concentration at the end of kisspeptin-10 infusion in relation to endogenous estradiol concentrations at 0 hours (i.e. before transdermal estradiol application). There was a strong positive correlation in controls (r^2=0.75, p=0.001) (Fig. 7.6). However, in NK3Ra-treated women, the LH response to kisspeptin-10 showed no such relationship (r^2=0.007, ns). Very similar results were obtained when the analysis was based on estradiol concentrations after 24 hours of patch administration (r^2=0.65, p=0.005 in controls; r^2=0.03, ns in NK3Ra-treated women).

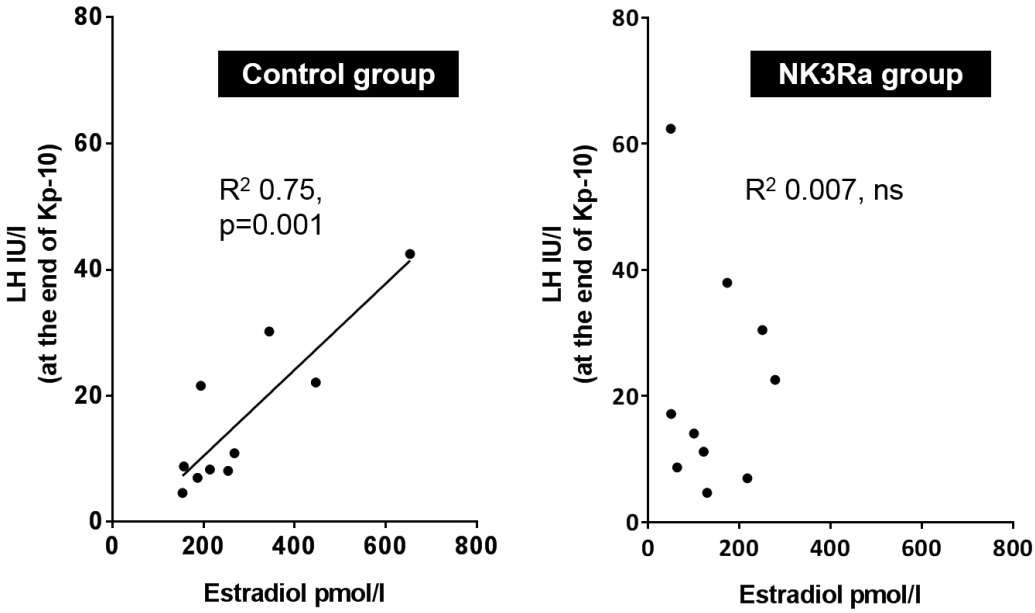

Figure 7.6 Correlation between endogenous estradiol and LH response to kisspeptin-10 in controls and NK3R antagonist treated women.

Kisspeptin-10 response on LH secretion is positively related to endogenous estradiol levels, whilst this correlation is not seen during NK3R antagonist treatment.

7.4.6 Interaction between NK3R antagonist and kisspeptin-10 in regulating LH pulsatility

LH pulse frequency increased from 0.7±0.1 pulses/hour in vehicle cycle to 1.0±0.1 pulses/hour during kisspeptin-10 infusion ($p<0.01$) (Fig. 7.7 A and B). NK3R antagonist reduced LH pulsatility to 0.5±0.1 pulses/hour ($p<0.05$ vs vehicle-infused controls), but administration of kisspeptin-10 to NK3Ra-treated women restored LH pulse frequency to that observed in kisspeptin-10-infused controls. Thus while NK3Ra slowed LH pulsatility in estrogen treated women, it did not affect the response to kisspeptin-10, indicating that kisspeptin effects are downstream of NKB signalling.

Secretory mass of LH per pulse was increased similarly during infusion of kisspeptin-10 compared with vehicle in both control ($p<0.05$) and NK3R antagonist treated women ($p<0.01$) (Fig. 7.7C). NK3Ra antagonist did not reduce LH secretory mass per pulse.

Consistent with increased LH pulse frequency, basal LH secretion decreased and pulsatile LH secretion increased during kisspeptin-10 infusion in the control group ($p<0.05$ vs vehicle; Fig 7.7 D and E). Basal LH secretion appeared lower in NK3Ra-treated women when compared to controls but there was no effect on pulsatile LH secretion. Kisspeptin-10 induced the same changes in NK3Ra treated women as in controls, with no change in basal and an increase in pulsatile LH secretion ($p<0.0001$).

The regularity of LH secretory pattern was assessed by approximate entopy (ApEn). Both kisspeptin-10 infusion and NK3Ra separately imposed greater orderliness (lower ApEn) in LH secretion ($p<0.05$; Fig 7.7F). This was increased further in NK3Ra- treated women during kisspeptin-10 infusion ($p<0.0001$ vs NK3Ra alone; Fig. 7.7F).

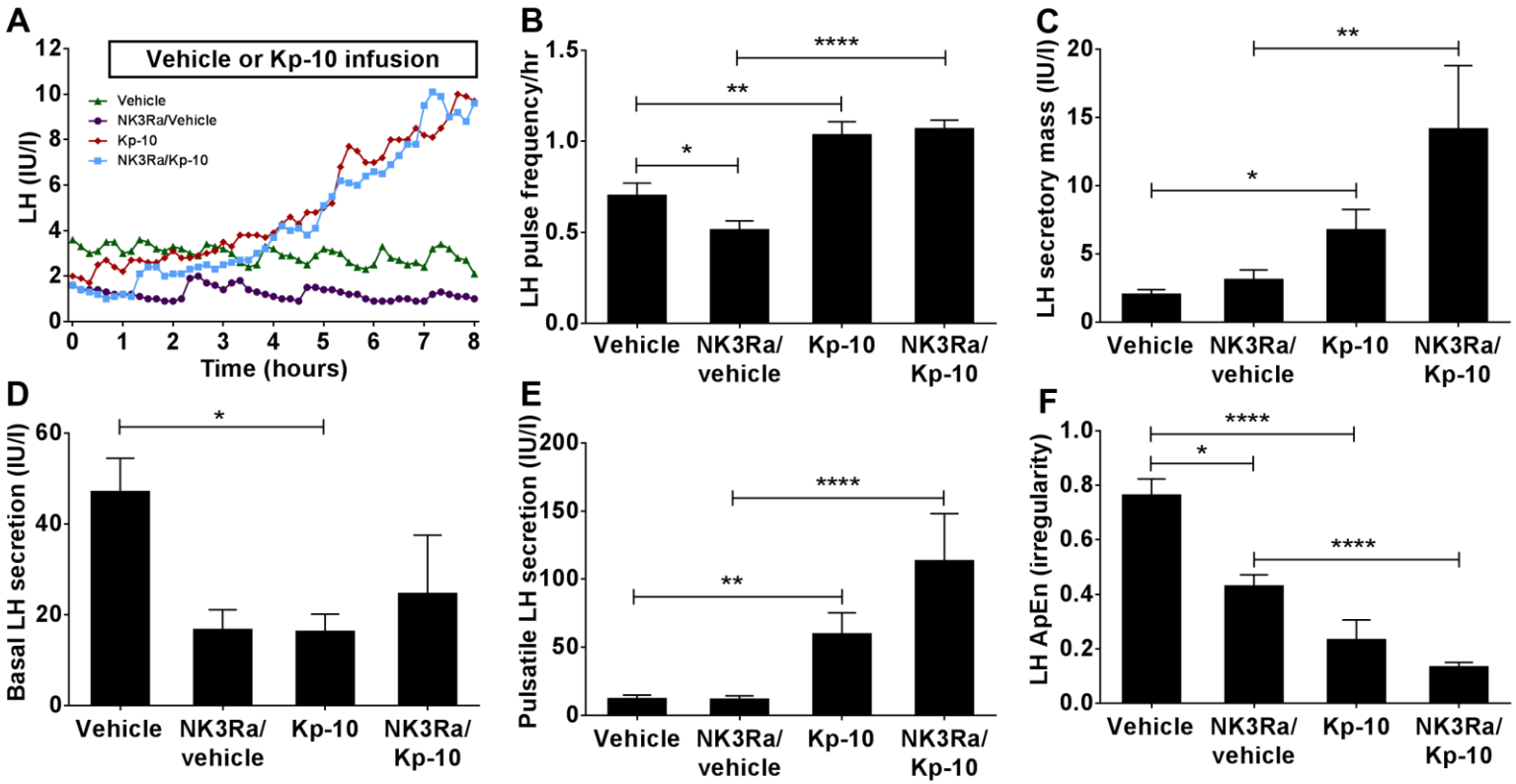

Figure 7.7 Analysis of 8 hour LH secretory pattern during vehicle and kisspeptin-10 in controls and NK3Ra-treated women.

(A) Illustrative LH pulse profile from one subject undergoing vehicle (*green triangles*), NK3R antagonist (*purple circles*), kisspeptin-10 (*red diamonds*) and NK3R antagonist followed by kisspeptin-10 (*blue squares*) treatment visits. Mean LH pulse frequency **(B)**, secretory mass of LH per pulse **(C)**, basal (nonpulsatile) LH secretion **(D)**, pulsatile LH secretion **(E)** and the relative orderliness/regularity of LH secretory pattern **(F)** during vehicle and kisspeptin-10 infusion with (n=9) and without (n=9) pre-treatment with NK3R antagonist. Mean ± SEM. *, $p<0.05$; **, $p<0.01$; ****, $p<0.0001$.

7.5 Discussion

In a model of LH modulation by estrogen administration in women, exogenous kisspeptin-10 stimulated LH secretion, the extent of which directly reflected estradiol concentrations. Pharmacological blockage of NKB-NK3R signalling slowed LH pulsatility and shortened the duration of kisspeptin-mediated LH secretion with 'sharpening' of the LH response and strikingly abolished the relationship between estradiol and LH response to kisspeptin. Taken together, these data support a central role for kisspeptin in the modulation of GnRH/LH secretion, and whilst NKB signalling is largely upstream of kisspeptin as previously reported (Young et al. 2013), both pathways interact in determining the timing and characteristics of estrogenic negative and positive feedback on LH secretion.

The stimulatory effect of exogenous kisspeptin on LH secretion in women is dependent on the sex steroid environment (Dhillo et al. 2007, Jayasena et al. 2011, Chan et al. 2012, George et al. 2012). This response is initially limited but increases markedly in the late follicular phase of the menstrual cycle when estradiol levels are rising (Dhillo et al. 2007, Jayasena et al. 2011, Chan et al. 2012). For most of the cycle, GnRH and thus LH secretion are inhibited by negative feedback from estradiol (and progesterone in the luteal phase), thus the low responsiveness to kisspeptin administration in previous studies is consistent with endogenous kisspeptin signalling being suppressed by steroid feedback. Conversely, the enhanced LH response in the later follicular phase may indicate the development of increased endogenous kisspeptin signalling in the lead up to the mid-cycle surge. This is supported by animal studies, where kisspeptin expression is highest following an estrogen challenge in the anteroventral periventricular nucleus (the site of positive estrogen feedback in rodents) in ovariectomised mice (Smith et al. 2005a) and at the time of GnRH/LH surge in sheep (Smith et al. 2006b), but is prevented by administration of kisspeptin receptor antagonist (Pineda et al. 2010, Smith et al. 2011). The present data suggests a role of estradiol in modulating LH response to kisspeptin-10 infusion, which persisted well beyond the pharmacokinetic clearance of the exogenous kisspeptin-10. The striking positive relationship between estradiol

concentration in the late follicular phase and the LH response to kisspeptin-10 infusion lends further support for the involvement of kisspeptin in estrogen feedback, as recently demonstrated for kisspeptin-54 (Narayanaswamy et al. 2015).

The present data demonstrate that NK3R antagonist treatment, in an environment of high estrogenic negative feedback, reduced LH secretion and pulse frequency, whilst in the presence of kisspeptin-10 had a stimulatory effect on the secretion of both gonadotropins, but with a shorter duration of LH response. Hitherto, NK3R antagonists have been demonstrated to suppress LH secretion in states of high LH output, such as in women with PCOS (George et al. 2016), or in the ovariectomised ewe and castrate monkeys (Fraser et al. 2015a, Li et al. 2015), and in intact female monkeys a delay of surge-like but no decrease in basal LH secretion was observed (Fraser et al. 2015b). Although it appears that the suppression of LH secretion by the present dose and regimen lasted only a few hours, this was sufficient to significantly lower estradiol concentrations after 5 days of NK3R antagonist treatment (i.e. prior to the estrogenic treatment) compared to controls. This is consistent with data in the early follicular phase of the menstrual cycle (Chapter 5), where marked suppression in follicle estradiol secretion was observed with no detectable changes in serum LH concentrations when measured once a day. Gonadotropin secretion was also unaffected by NK3R antagonism from the late follicular phase of the menstrual cycle (Chapter 6) as well as in postmenopausal women (Chapter 4). This suggests that in women irrespective of the sex-steroid milieu NKB action is not critical for maintaining circulating baseline LH concentrations, although it is involved in modulating the pulsatile pattern of GnRH/LH secretion. Indeed, in this model of estrogen-modulated LH secretion the frequency of LH pulses was reduced by the NK3R antagonist and in the early follicular phase the amount of LH secreted in between those pulses was suppressed (Chapter 5). This is however in contrast to men, where NK3R antagonist markedly suppressed LH and testosterone secretion by decreasing nonpulsatile GnRH/LH release whilst having no effect on the frequency of LH pulses (Chapter 3). This may reflect sexual dimorphism, where changes in GnRH/LH pulsatility throughout the menstrual cycle are a physiological feature for

normal reproductive function in women (Filicori et al. 1986) but not in men (Veldhuis et al. 2012).

In this thesis the role of NKB at the mid-cycle LH surge has been investigated in two models in healthy women. When administered in the late follicular phase, NK3R antagonist did not abolish the mid-cycle LH surge but did affects its timing, being more variable in the treatment compared to control cycle (Chapter 6), suggesting that NK3R antagonist perturbed the switch to positive estrogen feedback. However, no changes in peak estradiol secretion were detected and cycle length between the participants was variable, making it difficult to draw clear conclusions. We have therefore used exogenous estrogen exposure to standardise increased LH secretion, aiming to mimic the mid-cycle LH surge (Baird et al. 1995). NK3R antagonist had no effect on the timing of peak LH secretion in this model of estrogen administration, which is consistent with a lack of effect of NK3R antagonist on the estrogen induced LH surge seen in ovariectomised ewes (Li et al. 2015). The mechanisms critical for progression to positive estrogen feedback therefore appear to be largely independent of NKB but are dependent on kisspeptin, consistent with rodent neuroanatomical data (Smith et al. 2005a, Burke et al. 2006). A recent study using a different NK3R antagonist in normal women also showed a temporary suppression of LH levels lasting few hours post dosing, but no overall decrease in basal LH secretion following treatment throughout the follicular phase (Fraser et al. 2016). NK3Ra did, however, delay LH surge in some women, probably as a consequence of delayed pre-ovulatory estradiol rise, but the study did not assess the effect of neurokinin B antagonism at the time of the switch from negative to positive estrogen feedback, when NKB might be no longer critical (Fraser et al. 2016). Unlike in the present study, no effect of NK3Ra on FSH secretion was observed (Fraser et al. 2016).

Infusion of kisspeptin-10 can restore LH pulsatile secretion in men and women with inactivating mutations in NKB signalling, indicating that kisspeptin is functionally downstream of NKB in LH pulse generation (Young et al. 2013). This is supported by the inability of the NK3R agonist senktide to stimulate LH secretion in *Kiss1r* knockout mice (Garcia-Galiano et al. 2012). Consistent with this overall hierarchy,

NKB antagonism (active during kisspeptin-10 administration as half life of AZD4901 is 8.5 hours (Litman et al. 2014)) did not prevent the stimulatory effect of kisspeptin-10 infusion on LH secretion. NKB antagonist however shortened the LH response to kisspeptin-10, affecting its timing by reducing the variability of peak LH secretion, and disrupted the relationship between LH response and estradiol concentrations. These findings suggest a more complex interaction than a linear pathway between those neuropeptides at the time of the mid-cycle LH surge, but are also consistent with NK3R antagonist reducing endogenous kisspeptin stimulation of GnRH as a contribution to the observed effect.

LH pulse frequency increases in the late follicular phase, culminating in the mid-cycle LH surge (Yen and Tsai. 1971). Exogenous kisspeptin stimulates pulsatile LH secretion (George et al. 2011, George et al. 2013, Jayasena et al. 2013b, Young et al. 2013, Jayasena et al. 2014b), but its role as a potential contributor to positive estrogen feedback has not been previously investigated. In this study, the increase in LH secretion during kisspeptin-10 infusion included increased LH pulse frequency and mass-per-secretory pulse. This resulted in a larger proportion of total LH secretion occurring in pulsatile bursts, and the regularity of LH secretory pattern showed greater orderliness in the lead up to the stimulatory phase of response to estrogen. Deconvolution analysis also indicated changes in the nature of the pulsatile LH secretion following NK3R antagonist administration, with reduced basal secretion and ApEn, indicating a more orderly, slowed pattern of LH and by inference GnRH secretion. The increase in LH pulse frequency resulting from kisspeptin-10 infusion and the slowing in LH pulsatility with NK3R antagonist administration both increased the regularity and orderliness of LH secretion and may be the basis for the reduced variability in the timing of peak LH secretion as well as shortened duration of stimulated LH secretion in response to kisspeptin-10. Consistent with some aspects of our findings, estrogen-induced LH surges were preserved in ovariectomised NK3R antagonist-treated ewes, although the onset-to-peak time was delayed (Li et al. 2015). In sheep, the NK3R agonist senktide increased LH secretion, resembling ‘surge-like’ LH levels (Billings et al. 2010), whilst in monkeys, NK3R antagonist abolished LH surge, ovulation and subsequent

progesterone rise (Fraser et al. 2015b). While our data primarily indicate that NKB signalling is largely upstream of kisspeptin signalling in mediating estrogenic positive effects, it clearly has a modulatory role in determining the pattern and duration of GnRH secretion during estrogen positive feedback.

A stimulatory effect of kisspeptin alone on FSH has been minimal and inconsistent in previous studies (Dhillo et al. 2007, Jayasena et al. 2011, Chan et al. 2012, Skorupskaite et al. 2014), but was robustly demonstrated in this model and was not prevented by NK3R antagonist treatment. NK3R antagonist also increased FSH secretion, and markedly augmented stimulation by kisspeptin-10. Higher serum FSH concentrations were also observed with the NK3R antagonist in the early follicular phase of the menstrual cycle (Chapter 5). These findings with the NK3R antagonist are consistent with well-established data from animal models showing that high GnRH pulse frequency favours LH secretion while low pulse frequency favours FSH secretion (McNeilly et al. 2003), and that this is the main drive to follicular estrogen production, the reduction in both of which (and presumed reduced inhibin production, not determined here) is likely to have resulted in the observed increased FSH secretion. The differential effects of NK3Ra on FSH vs LH response to kisspeptin-10 are also similar to the effects in patients with NKB defects (Young et al. 2013). This does not explain the increased FSH secretion in response to kisspeptin with NK3R antagonist, when pulse frequency was not suppressed. This may indicate the importance of other aspects of pulsatile GnRH section in the differential regulation of LH and FSH, such as its regularity, as ApEn was decreased by both NK3R antagonist and kisspeptin, and further by the two in combination.

While the present study has clear strengths (the use of specific neurokinin-3 receptor antagonist, detailed LH pulse profiling and blinded pulse analysis), there are weaknesses. The sample size is small, and placebo was not administered to the control group receiving no NK3Ra. The limited LH suppression by the NK3R antagonist might be due to the small sample size and the dose of AZD4901 may be low compared to those used in animal studies, limiting the response (Fraser et al. 2015b). Statistical analyses included adjustment for alpha for multiplicity of comparisons but studies such as these should be regarded as mechanistic

explorations. Although our model of estrogen-stimulated LH secretion may be limited in representing the physiological LH surge, taken together with late follicular phase data, our results suggest a role for neurokinin B in mediating negative estrogen feedback, and the timing and pattern of the surge mode of GnRH/LH secretion in which kisspeptin is a key modulator.

In summary, using estrogen to standardise LH secretion in women to model the mid-cycle LH surge, we have shown that the increase in LH secretion by kisspeptin-10 infusion is related to estradiol exposure. We show that NK3R antagonist reduced LH pulsatility in healthy women. Assessment of the interaction between kisspeptin and NKB showed that the duration of kisspeptin-mediated LH secretion was shortened by the NK3R antagonist, and the quantitative relationship with estradiol exposure abolished. These data thus indicate that NKB pathways regulate GnRH/LH secretion in women, are predominantly upstream of kisspeptin signalling in mediating estrogen feedback, but modify this kisspeptin response. This extends our understanding of these critical events in human reproduction.

Chapter 8. General discussion and conclusions

In this thesis, the interaction and functional hierarchy between kisspeptin and neurokinin B in the control of human reproduction was investigated in states of fast and slow LH pulsatility and in varying sex-steroid environments in healthy men and women. Using pharmaceutical manipulation of NKB-KP-GnRH pathways we have demonstrated that neurokinin B is involved in the physiological regulation of reproductive function in men and women and that this is via the modulation of GnRH/LH pulsatility. Whilst neurokinin B signalling is predominantly proximal to kisspeptin, the hierarchy is more complex than simply linear in the control of human HPG axis, with the importance of the NKB pathway varying in different states. Primary results have been discussed in Chapters 3 to 7, with key themes emerging when the findings from those mechanistic studies are considered together.

8.1 Kisspeptin and neurokinin B interaction is complex

In patients with genetic mutations inactivating the TAC-TACR3 pathway, characteristically low LH pulse frequency was restored by exogenous kisspeptin administration, demonstrating for the first time in the human model that central neurokinin B signalling is functionally upstream of kisspeptin (Young et al. 2013). Whilst this is consistent with data from animal models (Billings et al. 2010, Corander et al. 2010, Navarro et al. 2011a, Ramaswamy et al. 2011, Garcia-Galiano et al. 2012), there are no previous studies investigating the hierarchy of kisspeptin and neurokinin B in the control of GnRH secretion in health rather than a disease setting, and in different states of LH pulsatility and sex-steroid environment across the reproductive cycle. This has been explored in this thesis with key findings summarised in Table 8.1.

	Men	Postmenopausal women	Premenopausal women
LH pulsatility	Slow	Fast	LH surge model
Sex-steroid feedback	Negative	Loss of sex-steroid stimulus	Negative then positive
NK3Ra response	Decreased LH and FSH Decreased T	Marginal LH decrease No FSH effect	Marginal decrease in basal LH No effect on peak LH Increased FSH
KP-10 response	Increased LH	No LH/FSH effect	Increased LH and FSH
KP-10 response in presence of NK3R antagonist	Increased LH	No LH/FSH effect	Shorter KP response Abrogated positive E2 feedback

Table 8.1 Summary table showing effects of kisspeptin and NK3R antagonism on gonadotropin secretion in different states of LH pulsatility and sex-steroid environments across human reproductive cycle.

Gonadotropin response to kisspeptin-10 and NK3R antagonist alone and in combination has been investigated in healthy men, postmenopausal women and in pre-menopausal women under estrogen administration to enhance LH secretion.

By using a consistent protocol of NK3R antagonism for 7 days and exogenous kisspeptin administration, we have shown that in men, postmenopausal women and in a model of LH surge in premenopausal women, there is a consistent functional hierarchy of those neuropeptides. This has been demonstrated at the level of both gonadotropin secretion and hypothalamic GnRH pulsatility, where a decreased LH pulse frequency with the NK3R antagonist was increased by kisspeptin infusion in healthy women. In men, LH secretion was decreased with the NK3R antagonist, but this did not affect an immediate stimulatory LH response to kisspeptin. Limited effect on LH secretion with kisspeptin administration has been shown in postmenopausal women previously (George et al. 2012) but its stimulatory role following potential LH suppression by NK3R antagonism has not been assessed. We have shown a different gonadotropin response in hypoestrogenic postmenopausal women, where the HPG axis was refractory to manipulation by kisspeptin and NK3R

antagonist. These data raise the possibility that with the loss of negative estrogen feedback in menopause, the system in not responsive to central manipulation. This is in keeping with the hypertrophy of kisspeptin and neurokinin B expressing neurones seen in the hypothalamus of postmenopausal women (Rance et al. 1990, Rance and Young. 1991) with similar findings in aging monkeys (Eghlidi et al. 2010). Supporting a role for neurokinin B in sex-steroid feedback, in a model of estrogen-induced LH secretion in premenopausal women, NK3R antagonist prevented the positive correlation between serum estradiol and LH response to kisspeptin, and furthermore shortened but did not abolish kisspeptin-stimulated LH secretion. Whilst being consistent with the overall hierarchy, this suggests an additional level of interaction between kisspeptin and neurokinin B at least in this model, whereby neurokinin B is having downstream effects modifying the kisspeptin response. Further evidence of a role for the NKB pathway in mediating estrogen feedback is provided by the increased variation in the timing of the LH surge in normal women administered NK3R antagonist in the late follicular phase.

This complexity is somewhat consistent with another study in healthy men, where stimulatory LH response to co-infusion of kisspeptin (KP-54) and neurokinin B itself was significantly lower than with kisspeptin alone, indicating potential inhibitory action of neurokinin B (Narayanaswamy et al. 2016c). Indeed, both stimulatory and inhibitory effects of exogenous neurokinin B administration have been reported in animal studies (Navarro et al. 2009, Billings et al. 2010, Ramaswamy et al. 2010, Navarro et al. 2011a). In a model of mid-cycle LH surge in women, the orderliness and regularity of LH secretion was greater with co-administration of kisspeptin-10 and NK3R antagonist than with either treatment alone, suggesting further the effects of NKB on kisspeptin signalling. Having investigated kisspeptin-neurokinin B interaction in regulating GnRH and thus gonadotropin secretion across different stages of reproductive health in men and women, this suggests that the interaction between these neuropeptides is complex with potential dual stimulatory and inhibitory actions of neurokinin B on downstream kisspeptin signalling, which may be dependant on sex-steroid environment.

8.2 NKB-KP pathway shows sexual dimorphism

In the present study, gonadotropin response to pharmacological blockade of neurokinin-3 receptor and exogenous kisspeptin administration was observed to be different between men and women, suggesting sexual dimorphism in NKB-KP pathways. Gender differences in their response to kisspeptin are well recognised in literature (Jayasena et al. 2011, Chan et al. 2012). This is further supported by the findings here, showing marked increase in LH with kisspeptin administration in men, but variable response in women, having no effect in estrogen deficient postmenopausal women in contrast to enhanced LH secretion in women under high estrogen exposure. Apart from some anatomical data, evidence for sexual dimorphism in neurokinin B signalling is less clear. In this thesis, LH secretion was assessed every 10 minutes for up to 8 hours post 6-7 days of NK3R antagonist administration in men, postmenopausal women and in premenopausal women during the follicular phase as well as in a model of estrogen-induced LH secretion. We have demonstrated that NK3R antagonist markedly suppressed LH secretion in men but this response in women was marginal. In the follicular phase and postmenopause, NK3R antagonist had no significant effect on LH secretion when sampled at single timepoints. Although overall LH secretion was lower with the NK3R antagonist treatment between 24 and 32 hours of estrogen exposure in a model LH surge, post hoc analysis indicated no significant differences in LH levels at any individual hourly timepoint over 8 hours, and no change in LH concentrations was observed throughout 7 days of NK3R treatment. These are novel findings in humans, indicating sexual dimorphism in neurokinin B signalling, but rather at variance with animal data showing greater abundance of neurokinin B expressing neurones in ewes than rams (Goubillon et al. 2000, Cheng et al. 2010) and earlier maturation of neurokinin B signalling in female rats (Ciofi et al. 2007). Our data in men are consistent with another study published during the preparation of this thesis, where a different NK3R antagonist (ESN364) decreased LH and testosterone secretion in healthy men (Fraser et al. 2016) with concordant results in castrate monkeys (Fraser et al. 2015a). ESN364 was also administered to healthy women throughout the follicular phase for 21 days, with the authors suggesting that baseline LH secretion

was also inhibited (Fraser et al. 2016). However, this effect was very short-lived showing significant LH inhibition at 4 and 6 hours only post dosing with no clear suppression following treatment throughout the follicular phase, but a delay in LH surge in some women likely as a consequence of delayed estradiol rise (Fraser et al. 2016). Furthermore, in intact female monkeys, NK3R antagonist administered at significantly higher doses did not show marked suppression in LH secretion throughout 35 day treatment but also delayed LH surges (Fraser et al. 2015a). Decrease in LH secretion in females has been demonstrated previously, but this was with central infusion of NK3R antagonist (MRK-08) in ovariectomised ewes (Li et al. 2015), and in women with PCOS, where LH hypersecretion is a part of the pathophysiology (George et al. 2016). Nevertheless, even if there is scope for manipulating LH secretion in women by the NK3R antagonist, this response is strikingly different to that observed in normal men in this and other published studies. Taken together, this suggests there may be gender different mechanisms by which neurokinin B modulates GnRH/LH secretion and this is consistent with sex differences observed in the KNDy neuronal network (Oakley et al. 2009). Inability to clearly manipulate LH secretion in women may reflect normal physiology where it is the cyclical change in GnRH/LH pulsatility that is prerequisite for normal gonadal function in women but not in men.

8.3 Kisspeptin and neurokinin B modulate GnRH pulsatility

It is well established that pulsatile GnRH secretion is pivotal for normal reproductive function (Herbison. 2015). Mechanisms for the GnRH pulse generator are not fully understood but kisspeptin and neurokinin B have been recently implicated as potential upstream modulators of this 'clock' (Herbison. 2015, Plant. 2015a). The role for kisspeptin in this has been demonstrated in normal men and women and in disease models (reviewed in (Skorupskaite et al. 2014, Clarke and Dhillo. 2016)). Administration of the same NK3R antagonist (AZD4901) to women with PCOS (George et al. 2016) and different NK3R antagonists (ESN364 and MRK-08) to ovariectomised ewes (Fraser et al. 2015a, Li et al. 2015) reduced the frequency of LH pulses, suggesting the role of neurokinin B in regulating GnRH/LH pulsatility at least in states of high LH output. However, there are no known studies exploring the role of neurokinin B and its interaction with kisspeptin in modulating pulsatile GnRH/LH release in health in humans.

The present study has shown for the first time that in normal men and women NK3R antagonist affected the pulsatile nature of LH secretion (a marker of GnRH release) but that this was variable across the groups studied (Table 8.2). This indicates that whilst neurokinin B acts centrally to modulate GnRH pulsatility, its interaction with GnRH is complex and influenced by additional factors. In keeping with published data, NK3R antagonist decreased LH pulse frequency but with the dose and regimen used here, this was only seen in women during estrogen-induced LH secretion and in those postmenopausal women with symptomatic hot flushes, but not in men or during other stages of the menstrual cycle. It is possible that there is a threshold to which a maximal suppression of pulse frequency can be achieved. Lower LH pulse frequency in men (0.5 pulses/hour in this study) may have precluded further pulse reduction by the NK3R antagonist in contrast to increasing LH pulsatility throughout the late follicular phase with observed decrease in LH pulse frequency from 0.7 pulses to 0.5 pulses/hour in this model of the LH surge. However, this does not seem to be an absolute determinant for LH pulse modulation since in the early follicular phase mean LH pulse frequency was 0.7/hour and in all postmenopausal women irrespective of their flush status was approximately 0.9 pulses/hour. Estrogen

feedback appears to be at least one candidate affecting neurokinin B modulation on pulsatile GnRH/LH secretion. In a model of LH surge, NK3R antagonist slowed LH pulsatility and abolished the correlation between estradiol and LH response to kisspeptin, which is in contrast to the absence of effect on LH pulse frequency observed in women with lower estradiol levels in the early follicular phase and in hypoestrogenic postmenopausal women in a group as a whole. Sex-steroids are known to regulate LH pulse frequency (Veldhuis et al. 2008), thus the fall in serum testosterone in men and estradiol in women is likely to have counteracted any suppressive effect of NK3R antagonism on pulsatile LH secretion. Furthermore, inability to manipulate LH pulse frequency in men and menopausal women may reflect normal physiology in that changes in LH pulse frequency are not essential for normal testicular function in men (Veldhuis et al. 2012) and the prolonged estrogen deficiency in postmenopausal women may have affected its role, unlike its key role in regulating menstrual cycle in women of reproductive age (Filicori et al. 1986).

In addition to the frequency of LH pulses most commonly assessed in previous studies, other parameters of the pattern of LH/GnRH secretion were analysed in this thesis (Table 8.2). The amount of LH secreted in between the pulses (basal secretion) was affected most consistently by the NK3R antagonist, being reduced in men, postmenopausal women and in women in the early follicular phase with a non statistically significant decrease in a model of the mid-cycle LH surge (when pulse frequency was reduced). In men, the total amount of LH released in pulsatile bursts was also reduced by the NK3R antagonist, and LH secretory pattern was more regular (decreased ApEn), which were sufficient to induce a testicular response with markedly decreased testosterone secretion, despite no observed changes in LH pulse frequency. This suggests that these parameters of pulsatile LH/GnRH release are important in mediating Leydig cell function. Similarly, ovarian estradiol secretion was decreased and follicle development was arrested during early follicular phase administration of NK3R antagonist, where basal (nonpulsatile) LH secretion was the only parameter of GnRH/LH secretory pattern affected. Increased orderliness of GnRH/LH secretion was a relatively consistent effect of NK3R antagonism, indicating a role for NKB signalling in reducing that orderliness in its stimulatory

effects on the regulation of GnRH secretion. Taken together, manipulation of pulsatile GnRH/LH secretion by the NK3R antagonist in normal men and women and the downstream effects on gonadal function, suggest a key role of neurokinin B in modulating GnRH pulsatility and thus reproductive function in humans but with the details of the impact on GnRH secretion varying between men and women and between different physiological states.

Group	**Men**	**Postmenopausal women-all**	**Postmenopausal women- with hot flushes only**	**Premenopausal women- early follicular phase**	**Premenopausal women- LH surge model**		
Treatment/Pulsatility parameter	*NK3Ra*	*NK3Ra*	*NK3Ra*	*NK3Ra*	*NK3Ra*	*KP-10*	*NK3Ra and KP-10*
LH pulse frequency	⟷	⟷	↓	⟷	↓	↑	↑
LH mass per pulse	⟷	↑	↑	⟷	⟷	↑	↑
Basal LH	↓	↓	↓	↓	⟷	↓	⟷
Pulsatile LH	↓	⟷	⟷	⟷	⟷	↑	↑
ApEn (irregularity)	↓	⟷	⟷	⟷	↓	↓	↓

Table 8.2 Effects on NK3R antagonism, kisspeptin-10 infusion and their combination on various parameters of pulsatile nature of LH and by inference GnRH secretion in healthy men and women in different stages of reproductive life.

8.4 Strengths and limitations

The studies described in this thesis were proof-of-principal mechanistic studies in human volunteers, exploring the physiology of hypothalamic regulation of reproductive function. We have used the same protocol of administration of a specific neurokinin-3 receptor antagonist (AZD4901) and exogenous kisspeptin-10 (iv bolus or an infusion) across different stages of reproductive health reflecting states of high and low LH pulsatility and various sex-steroid milieu. Effects on reproductive hormone secretion and detailed LH pulse profiling with blinded pulse analysis was performed, forming a comprehensive series of studies to support the role of NKB and its complex interaction with kisspeptin in regulating human reproductive function through the modulation of pulsatile nature of GnRH secretion.

It is important to acknowledge that the sample size is small in the present studies. A sample size of up to 10 volunteers is commonly used in such mechanistic studies by others (Dhillo et al. 2005, Dhillo et al. 2007, Jayasena et al. 2011) and in previous work by our group (George et al. 2011, George et al. 2012), demonstrating significant changes in reproductive hormone secretion and LH pulsatility. However, small sample size may have affected statistical power to detect subtle effects of NK3R antagonism on LH secretion in women. Furthermore, limited LH suppression and variable effects on LH pulse frequency by the NK3R antagonist might be due to 80mg of AZD4901 being at the bottom of the dose response curve, this was the lowest dose that had an effect on LH secretion in women with PCOS (George et al. 2016). It has to be noted that marked effects on LH pulsatility and serum concentrations achieved in animal studies were often with central administration of NK3R antagonist and with larger doses (Fraser et al. 2015a, Li et al. 2015). In addition, there are anatomical and therefore likely physiological differences in NKB-KP pathway in animals compared to humans, making direct comparisons difficult, and there are to date limited human data on the interaction between kisspeptin and neurokinin B in regulating the HPG axis. Peripherally administrated drugs in this study may not have fully reached the hypothalamic regions of their action, although

KNDy neurones send projections to the median eminence and the arcuate nucleus, which have an incomplete blood-brain-barrier (Rodriguez et al. 2010).

Compliance with taking the NK3R antagonist was not formally assessed, such as by using drug diaries. Participants were asked to return drug bottles after the study, and although the tablet count was correct this is not a reliable indicator alone. We have no reason to suspect non-compliance, as reproductive hormone profiles during treatment were consistent with the overall pattern of effect between individuals.

The present study was an open label one and placebo was not administered to the control group receiving no NK3R antagonist. This may have particularly influenced demonstration of reduction in flush symptoms by the NK3R antagonist in postmenopausal women. However, paired data were obtained for the control period and administration of NK3R antagonist in most studies, and the order of those was randomised (in women in early and late follicular, luteal phase and model of pre-ovulatory LH surge) as was the infusion of kisspeptin-10 or saline, using a sealed envelope technique. The same operator performed all ultrasound assessments throughout the study. Reproductive hormone samples were analysed in duplicate by the operator blinded to treatment allocation, and deconvolution analysis was also blinded to treatment allocation.

We have used a model of exogenous estradiol administration previously demonstrated to induce LH secretion at 48 hours mimicking positive estrogen feedback by Baird and colleagues (Baird et al. 1995). This model may not fully replicate the physiological system of the pre-ovulatory state. Nevertheless, it allowed us to standardise estrogen exposure in women and gain some insight into neurokinin B and kisspeptin interaction in positive estrogen feedback, which is otherwise challenging to study in normal women with variable timing and extent of follicle development and estrogen production. Although the LH increase at 48 hours might be in part secondary to loss of negative estrogen feedback with exogenous estradiol removal at 32 hours, we have shown that continuing estrogen administration at 48 hours had no effect in serum LH concentration at that time. This is therefore suggestive of positive estrogen feedback triggering LH secretion in our model.

8.5 Clinical application of NKB-KP manipulation and future work

GnRH analogues are used extensively in clinical practice mainly to downregulate gonadal function in sex-steroid dependent disorders, e.g. in breast and prostate cancer, endometriosis, uterine fibroids, and in assisted conception. Although effective, GnRH analogues suppress gonadal steroids to near castrate levels, giving rise to menopausal-like side effects, such as hot flushes, loss of libido and reduced bone mass density. Classification of reproductive endocrine conditions into the states of high (PCOS, menopause, precocious puberty), low (delayed puberty, hypothalamic amenorrhoea, hypogonadism in diabetes) and normal (endometriosis, fibroids, prostate hypertrophy) GnRH/LH pulsatility makes manipulation of NKB-KP pathways a novel therapeutic approach in managing various reproductive endocrine states (Fig. 8.1).

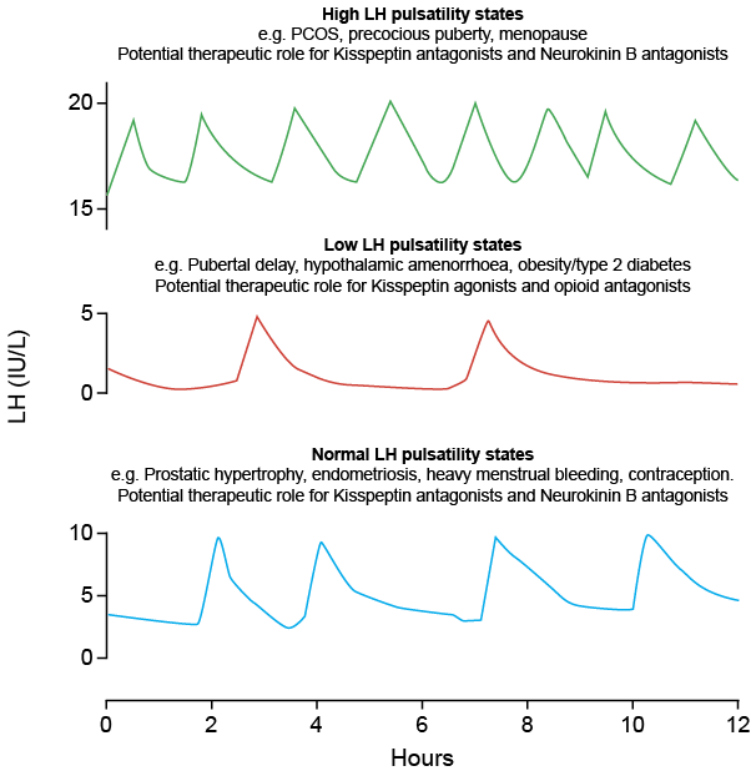

Figure 8.1 Potential therapeutic application of manipulation of kisspeptin and neurokinin B based LH secretion.

Kisspeptin and neurokinin B antagonists have therapeutic potential to decrease LH pulsatility in health and in reproductive endocrine disorders where LH pulse frequency is high, whilst kisspeptin and neurokinin B agonists and opioid antagonists may increase pulsatile LH secretion where it is low. Adapted from Skorupskaite et al. (2014).

8.5.1 Manipulation of NKP-KP pathways to suppress HPG axis

Manipulation of KNDy neurones to supress the stimulatory action of kisspeptin and neurokinin B by their receptor antagonists, and to stimulate the inhibitory action of dynorphin by its agonists, offers therapeutic potential to reduce gonadotropin and thus sex-steroid secretion where GnRH/LH pulsatility is normal or high (Fig. 8.1).

Therapeutic potential in states of normal GnRH/LH pulsatility

In the present study, NK3R antagonist in healthy women halted ovarian follicle development and pre-ovulatory estradiol rise, thereby delaying the mid-cycle LH surge and ovulation. Our data suggest potential therapeutic application of neurokinin B antagonism in non-steroidal contraception, being specifically advantageous in women where estrogen is contraindicated. Moreover, a delay in ovulation for the duration of treatment may be also applied as pro-contraceptive. Similar effects were observed with a different NK3R antagonist (ESN364) administration for 21 days delaying positive estrogen feedback and LH surge, although, in contrast to our data, no changes in ovarian follicle growth were demonstrated (Fraser et al. 2016). Importantly, we have shown that after treatment was discontinued, follicle development resumed with evidence of normal ovulation and luteal function. Luteal function was consistently unaffected by NK3R antagonist administration in the late follicular and luteal phases. However, possible effects on endometrium remain to be established since NKB-NK3R pathway is expressed in human uterine epithelium (Cejudo Roman et al. 2012). Indeed, uterine stromal atrophy with inactive glands was observed with NK3R antagonism (ESN364) in monkeys but those changes were reversible after treatment discontinuation (Fraser et al. 2015a) and it is unclear whether these findings merely reflect the changed endocrine environment. Clinical application of NK3R antagonism might be limited by the resulting lack of progesterone exposure and potential adverse endometrial effects associated with it. In the present study, treatment was for 7 days only but whether such effects would persist with longer NK3R antagonist administration is yet to be determined.

Potentially more subtle decrease in LH than FSH secretion by antagonism of kisspeptin and neurokinin B signalling contrasts with profound suppressive effects of GnRH analogues, which decrease gonadotropin and sex steroid secretion to near

castration levels with consequent side effects, including vasomotor symptoms, loss of libido and decreased bone mineral density (Roseweir et al. 2009). Whilst complete downregulation of gonadal steroids is necessary in some conditions, such as prostate cancer, partial suppression is more desirable in sex-steroid dependent disorders, such as benign prostatic hyperplasia, endometriosis, uterine fibroids and heavy menstrual bleeding. Estradiol levels were above castrate levels with the NK3R antagonist in our female studies and those reported by others (Fraser et al. 2016), avoiding the unwanted side effects of estrogen deficiency. Clinical advantage of incomplete suppression of the HPG axis is further illustrated in the management of endometriosis and uterine fibroids, where selective progesterone receptor modulators and/or GnRH analogues with sex-steroid add back are often used. Furthermore, the NKB-NK3R pathway is upregulated in human uterine fibroids (Canete et al. 2013) and a randomised placebo controlled trial is currently underway investigating NK3R antagonism in in this setting. The thinner endometrium observed with NK3R antagonist administration in the early follicular phase in our study is a foundation for assessment of menstrual blood loss, which could translate into therapies to reduce heavy menstrual bleeding.

A similar therapeutic approach might support the use of kisspeptin and neurokinin B inhibition in benign prostatic hypertrophy. In men, NK3R antagonist suppressed testosterone levels and this was more profound in men compared to estradiol reduction in women. Testosterone concentrations were markedly reduced after 24 hours of treatment but then remained at the lower end of normal for the duration of NK3R antagonist administration, with one man reported reduced libido, which recovered after treatment. Similarly, kisspeptin analogues were shown to decrease testosterone into the castration range in healthy men (Maclean 2013, Scott 2013), potentially limiting the clinical use of antagonism of the actions those neuropeptides.

Therapeutic potential in states of high GnRH/LH pulsatility

It is a novel observation that postmenopausal women administered NK3R antagonist reported subjective reduction in their hot flush frequency and severity. Indeed, LH pulses were shown to coincide with hot flushes (Casper et al. 1979, Oakley et al. 2015), the expression of kisspeptin and neurokinin B is unregulated postmenopause

(Rance. 2009), and in premenopausal women 30-minute infusion of neurokinin B induced the sensation of hot flushes (Jayasena et al. 2014c). We have shown that NK3R antagonist reduced the frequency of LH pulses in those postmenopausal women reporting flush symptoms. Taken together this data support a link between GnRH/LH pulsatility and vasomotor symptoms (Casper et al. 1979, Rance et al. 2013) and NK3R antagonism as a potential therapeutic approach. We do appreciate lack of placebo in the present study but this warrants further exploration in the form of a randomised placebo controlled trial and an objective measure of hot flushes, such as by using sternal conductance monitoring.

In the present study, both exogenous kisspeptin and NK3R antagonist have been shown to increase and decrease LH pulse frequency, respectively, and to affect other parameters of pulsatile nature of LH/GnRH secretion. Slowing down GnRH pulse frequency, which preferentially determines LH secretion (McNeilly et al. 2003), by kisspeptin and/or neurokinin B receptor antagonists may selectively suppress hypersecretion of LH seen in PCOS. Indeed, a recent phase 2 trial administered the same NK3R antagonist (AZD4901) to anovulatory hyperandrogenaemic women with PCOS, demonstrating suppression in LH pulse frequency and subsequent decrease in serum LH and testosterone levels (George et al. 2016). The rate of ovulation was equivalent between the control and treatment groups, and it was not possible to distinguish if this was a spontaneous or potentially treatment-induced ovulation (George et al. 2016). A randomised controlled trial administering higher NK3R antagonist doses for longer in women with PCOS is currently underway to determine effects of neurokinin B antagonism on resumption of folliculogenesis and menstruation as well as its role in the treatment of hirsutism. Although the inhibitory role of opioids on GnRH and LH pulsatility is well known chronic opioids exposure in the treatment of reproductive disorders remains controversial.

8.5.2 Manipulation of NKB-KP pathways to stimulate HPG axis

Manipulation of KNDy neurones to enhance the stimulatory action of kisspeptin and potentially neurokinin B by their cognate agonists, and to suppress the inhibitory tone of dynorphin by its antagonists, offers therapeutic potential to increase gonadotropin secretion where it is low (Figure 8.1).

Therapeutic potential in states of low GnRH/LH pulsatility

In addition to the stimulatory role of kisspeptin on gonadotropin release in healthy men and women, kisspeptin has been shown to increase LH secretion and pulse frequency in women with hypothalamic amenorrhoea (Jayasena et al. 2014b), hypogonadal men with diabetes (George et al. 2013) and in patients bearing inactivating TAC3/TACR3 mutations (Young et al. 2013), but with suboptimal effects on gonadal steroid secretion.

In women with hypothalamic amenorrhoea twice-weekly administration of kisspeptin (KP-54 6.4 nmol/kg) maintained gonadotropin secretion for 8 weeks with no significant increase in estradiol levels, suggesting that ovarian activity remained quiescent, as confirmed by unchanged follicle size and endometrial thickness on ultrasound scan (Jayasena et al. 2010). FSH response to kisspeptin has been minimal and inconsistent in previous studies (Dhillo et al., 2007, Jayasena et al., 2011, Chan et al., 2012), and may account for this lack of androgen aromatisation. An inhibitory action of NKB has been suggested following the observation that co-infusion of NKB and kisspeptin had lesser stimulatory response on LH secretion than kisspeptin alone (Narayanaswamy et al. 2016c). Dual administration of kisspeptin and NK3R antagonist may stimulate LH and FSH secretion sufficiently to increase estradiol secretion in women with hypothalamic amenorrhoea. Kisspeptin may need to be administered more frequently, for longer and by a more potent route to achieve longer-term effects on gonadal function. Indeed, intravenous infusion of the highest dose of kisspeptin-54 (1.0 nmol/kg/hour) in those women for 10 hours increased pulsatile LH secretion with serum estradiol continuing to increase for the duration of the infusion (Jayasena et al. 2014b). However, a gradual decline in LH secretion was observed after 5 hours of kisspeptin infusion (Jayasena et al. 2014b), as it was with twice daily subcutaneous injections for 2 weeks (6.4 nmol/kg) (Jayasena et al. 2009),

indicating possible tachyphylaxis. The opioid antagonist, naltrexone, has been administered in women with hypothalamic amenorrhoea, where it increased LH pulse amplitude and circulating estradiol levels and restored menstruation in some patients (Genazzani et al. 1995a). The role of opioid antagonism in stimulating gonadotropin secretion in other cases of abnormally low LH secretion remains to be elucidated.

Lack of gonadal steroid effect by exogenous kisspeptin has also been demonstrated in other causes of hypogonadotropic hypogonadism. In men and women with defects in neurokinin B pathway, testosterone and estradiol secretion did not reach physiological levels, respectively, despite increased LH pulse frequency by kisspeptin administration (KP-10 1.1 nmol/kg/hour for 12 hours) (Young et al. 2013). Whilst kisspeptin-10 normalised serum testosterone in diabetic hypogonadal men, baseline testosterone levels were above castrate compared to other studies (George et al. 2013). Therapeutic benefits of NKB-KP pathway manipulation in patients with low GnRH/LH pulsatility will be determined by the ability to bring about gonadal function without desensitisation when used for a longer period of time.

The role of neurokinin B in patients with abnormally low LH secretion has not been investigated, which is likely to stem from lack of stimulatory or inhibitory effect of neurokinin B on reproductive hormone secretion seen in healthy men and women (Jayasena et al. 2014c). In animal models kisspeptin and neurokinin B has been shown to advance pubertal maturation (Navarro et al. 2004b, Plant et al. 2006, Nakahara et al. 2013) but neither of these neuropeptides have been administered to children with delayed puberty. Nevertheless, pubertal induction with kisspeptin and neurokinin B may be more physiological in stimulating LH pulses compared with sex hormone therapy used currently in girls and boys with delayed puberty.

Hyperprolactinaemia inhibits pulsatile GnRH/LH release and results in hypogonadotropic anovulation and infertility, both of which are reversed by pulsatile GnRH infusion (Bouchard et al. 1985, Lecomte et al. 1997), suggesting a hypothalamic action of prolactin. Whilst only few GnRH neurones express prolactin receptors (Grattan et al. 2007), they are present on kisspeptin neurones (Kokay et al.

2011). Prolactin-induced anovulation in mice is associated with diminished kisspeptin expression in the AVPV and the arcuate nucleus (Sonigo et al. 2012), suggesting that prolactin mediates its actions upstream of GnRH and via kisspeptin signalling. Indeed, intraperitoneal administration of Kp-10 restored suppression of gonadotropin secretion and ovarian acyclicity in hyperprolactinaemic mice (Sonigo et al. 2012). There are no published data on kisspeptin administration in patients with hyperprolactinaemia, but it may be an alternative therapy to restore fertility in those who are resistant or intolerant to dopamine agonists.

Kisspeptin therapy has the potential to finesse *in vitro* fertilization (IVF) techniques. Kisspeptin-54 increased LH secretion sufficiently to induce oocyte maturation in a FSH/GnRH antagonist superovulation protocol with achievement of live term births (Jayasena et al. 2014a). Furthermore, the LH response to exogenous kisspeptin is of much shorter duration that following hCG administration, reducing the risk of life-threatening ovarian hyperstimulation syndrome (OHSS). Indeed, women at risk of OHSS, who received kisspeptin-54 following ovarian stimulation, developed no moderate, severe or critical OHSS (Abbara et al. 2015). These are preliminary but promising insights into alternative agents used in assisted reproduction and a large randomised controlled trial is required to determine potential advantages and disadvantages over current approaches.

Nevertheless, the discovery of kisspeptin and neurokinin B has revolutionised our understanding of the neuroendocrine signals controlling reproductive axis. The potential for translational application of kisspeptin and neurokinin B analogues is being explored in several clinical trials and it is likely that the manipulation of NKB-KP signalling will soon be used in the treatment of reproductive disorders and infertility.

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Chapter 10. Appendix- published manuscripts

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human reproduction update

The kisspeptin-GnRH pathway in human reproductive health and disease

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BACKGROUND: The discovery of kisspeptin as key central regulator of GnRH secretion has led to a new level of understanding of the neuroendocrine regulation of human reproduction. The related discovery of the kisspeptin-neurokinin B-dynorphin (KNDy) pathway in the last decade has further strengthened our understanding of the modulation of GnRH secretion by endocrine, metabolic and environmental inputs. In this review, we summarize current understanding of the physiological roles of these novel neuropeptides, and discuss the clinical relevance of these discoveries and their potential translational applications.

METHODS: A systematic literature search was performed using PUBMED for all English language articles up to January 2014. In addition, the reference lists of all relevant original research articles and reviews were examined. This review focuses mainly on published human studies but also draws on relevant animal data.

RESULTS: Kisspeptin is a principal regulator of the secretion of gonadotrophins, and through this key role it is critical for the onset of puberty, the regulation of sex steroid-mediated feedback and the control of adult fertility. Although there is some sexual dimorphism, both neuroanatomically

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and functionally, these functions are apparent in both men and women. Kisspeptin acts upstream of GnRH and, following paracrine stimulatory and inhibitory inputs from neurokinin B and dynorphin (KNDy neuropeptides), signals directly to GnRH neurones to control pulsatile GnRH release. When administered to humans in different isoforms, routes and doses, kisspeptin robustly stimulates LH secretion and LH pulse frequency. Manipulation of the KNDy system is currently the focus of translational research with the possibility of future clinical application to regulate LH pulsatility, increasing gonadal sex steroid secretion in reproductive disorders characterized by decreased LH pulsatility, including hypothalamic amenorrhoea and hypogonadotropic hypogonadism. Conversely there may be scope to reduce the activity of the KNDy system to reduce LH secretion where hypersecretion of LH adds to the phenotype, such as in polycystic ovary syndrome.

CONCLUSIONS: Kisspeptin is a recently discovered neuromodulator that controls GnRH secretion mediating endocrine and metabolic inputs to the regulation of human reproduction. Manipulation of kisspeptin signalling has the potential for novel therapies in patients with pathologically low or high LH pulsatility.

Key words: kisspeptin / kisspeptin-neurokinin B-dynorphin / GnRH / LH pulsatility

Introduction

Since its discovery, hypothalamic secretion of GnRH has been robustly established as the key pathway that initiates and controls reproductive function. Whilst the pivotal central role played by GnRH remains undisputed, a number of functional limitations of the GnRH neuronal network have been identified. For example, in rats, GnRH neurones lack estrogen receptor (ER)-alpha (Herbison and Theodosis, 1992), the principal ER, thus suggesting the need for an intermediary signalling pathway mediating gonadal feedback.

It was only a decade ago that the discovery of the obligate role of kisspeptin in human puberty revolutionized current understanding of the neuroendocrine regulation of human reproduction (de Roux *et al.*, 2003; Seminara *et al.*, 2003). Kisspeptin, a hypothalamic peptide coded by the KiSS1 gene, is a novel neuromodulator that acts upstream of GnRH, and is sensitive to sex steroid feedback and metabolic cues. Kisspeptin is now recognized as a crucial regulator of the onset of puberty, the regulation of sex hormone-mediated secretion of gonadotrophins, and the control of fertility (Pinilla *et al.*, 2012). The related discovery of a reproductive role for neurokinin B has stimulated further interest in the field. The same functional neuronal network secretes kisspeptin and neurokinin B—now called kisspeptin-neurokinin B-dynorphin (KNDy) neurones as they also co-secrete dynorphin, a well-established opioid inhibitor (Goodman *et al.*, 2007). Exogenous kisspeptin has been administered to healthy volunteers and a limited number of patients, with a view to restoring reproductive function in certain conditions.

In this review, we summarize current understanding of the physiological regulation of GnRH pulse frequency by kisspeptin, and appraise the clinical relevance of the discoveries of kisspeptin and neurokinin B. The focus will predominantly be on human findings, using animal data where human studies are lacking but where there is direct translational potential.

Methods

A systematic literature search was performed using PUBMED for all English language articles published up to January 2014 using the terms 'kisspeptin' and 'reproduction'. The search was performed without limitations by species although subsequent priority was given to human studies, where available. The initial search identified 390 manuscripts, which were used as background material for the review. In addition, the reference lists of all relevant original research articles and reviews were examined and selected if judged to be relevant. Relevant abstracts from recent scientific meetings were included in the review.

Discovery of the kisspeptin and KNDy neuronal network

KISS1 gene, peptide and its receptor

KISS1, the gene encoding kisspeptins, was originally identified in 1996 as a suppressor of metastasis in human malignant melanoma (Lee *et al.*, 1996). As it was discovered in Hershey (PA, USA), the gene was named after the famous chocolate 'Kisses' produced in the town. The SS in *KiSS1* acknowledges that it is a 'suppressor sequence'.

The *KISS1* gene is localized to chromosome 1q32 and has four exons, the first two of which are not translated (West *et al.*, 1998). The gene encodes the precursor 145 amino acid peptide, which is cleaved to a 54 amino acid protein (West *et al.*, 1998). To acknowledge its metastasis inhibitory properties, the 54 amino acid transcript was named 'metastin' (Ohtaki *et al.*, 2001). This can be further cleaved to 14, 13 and 10 amino acid peptides. The 54 amino acid and the shorter peptides belong to the RF amide group of peptides, sharing the C-terminal sequence of Arg-Phe-NH_2, and are now collectively referred to as kisspeptins (Kotani *et al.*, 2001).

In 2001, kisspeptin was identified as a ligand for the orphan G-protein coupled receptor 54 (GPR54), which was first described in the rat brain and subsequently in human (then named AXOR12 and hOT7T175) (Lee *et al.*, 1999; Muir *et al.*, 2001; Ohtaki *et al.*, 2001), now termed KISS1R (Gottsch *et al.*, 2009). *KISS1R* maps to chromosome 19p13.3 and includes five exons, encoding a 398 amino acid protein with seven hydrophobic trans-membrane domains (Muir *et al.*, 2001). It has an amino acid sequence close to that of the galanin receptor family (40% identity), although it does not bind either galanin or galanin-like peptide (Lee *et al.*, 1999). Upon binding by kisspeptin, KISS1R activates phospholipase C and recruits secondary intracellular messengers, inositol triphosphate and diacylglycerol, which in turn mediate calcium release and protein kinase C activation to mediate kisspeptin's function (Muir *et al.*, 2001; Liu *et al.*, 2008; Constantin *et al.*, 2009). Activation of Kiss1r results in a biphasic increase in intracellular calcium, with a rapid increase followed by a more sustained second phase (Min *et al.*, 2014). To maintain this second phase and therefore sustain signalling, kisspeptin receptor trafficking involving internalization, recycling and recruitment from an intracellular pool, is required (Min *et al.*, 2014). Without

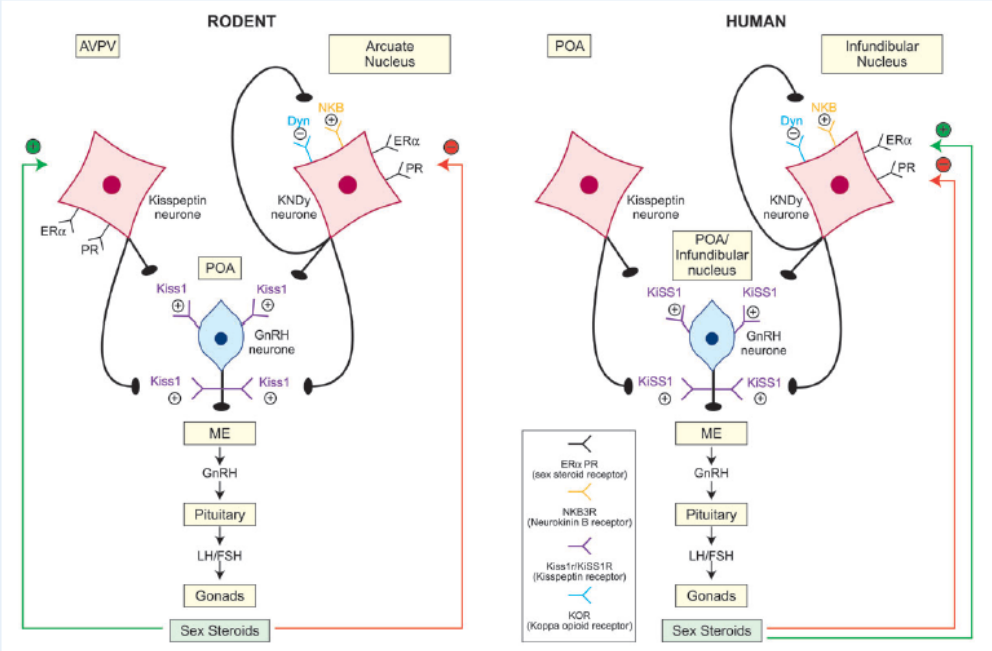

Figure 1 Schematic diagram showing the neuroanatomy of the kisspeptin-GnRH pathway and the relationship between KNDy neurones and GnRH neurones in humans and rodents. Kisspeptin signals directly to the GnRH neurones, which express kisspeptin receptor. The location of kisspeptin neurone populations within the hypothalamus is species specific, residing within the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus in rodents, and within the preoptic area (POA) and the infundibular nucleus in humans. Kisspeptin neurones in the infundibular (humans)/ arcuate (rodents) nucleus co-express neurokinin B and dynorphin (KNDy neurones), which via neurokinin B receptor and kappa opioid peptide receptor autosynaptically regulate pulsatile kisspeptin secretion, with neurokinin B being stimulatory and dynorphin inhibitory. Negative (red) and positive (green) sex steroid feedback is mediated via distinct kisspeptin populations in rodents, via the AVPV and the arcuate nucleus, respectively. In humans KNDy neurones in the infundibular nucleus relay both negative (red) and positive (green) feedback. The role of the POA kisspeptin population in mediating sex steroid feedback in humans is incompletely explored. ME, median eminence; +, stimulatory; −, inhibitory; ERα, estrogen receptor alpha; PR, progesterone receptor; Kiss1 / KiSS1, kisspeptin; NKB, neurokinin B; Dyn, dynorphin.

receptor trafficking, the kisspeptin receptor undergoes desensitization following an initial acute phase (Min *et al.*, 2014). Since the discovery of kisspeptin-KISS1R signalling, many different terms have been used to describe its components. The nomenclature used in this review for kisspeptin and its receptor will be that recently recommended by Gottsch *et al.* (2009).

Functional neuroanatomy of kisspeptin signalling

GnRH neurones extend from the preoptic area through to the infundibular nucleus (homologue to the arcuate nucleus in other species) of the hypothalamus in humans, whereas in rodents GnRH neurones reside predominantly in the preoptic area (Lehman *et al.*, 1986; Schwanzel-Fukuda and Pfaff, 1989; Clifton and Steiner, 2009) (Fig. 1). GnRH axons project from these nuclei to the median eminence, where GnRH is secreted into the portal circulation in a coordinated and pulsatile manner. Similarly, kisspeptin neurones are located in the rostral preoptic area and the infundibular nucleus in the human hypothalamus (Rometo *et al.*, 2007; Hrabovszky *et al.*, 2010). The anatomical distribution of kisspeptin neurones and their appositions with other hypothalamic endocrine networks are described below. Areas of incongruity between data from human studies and those carried out in other species are also highlighted.

Kisspeptin neurone localization in humans

Initial studies of the neuroanatomical distribution of kisspeptin neurones in the human brain carried out in autopsy samples from premenopausal and post-menopausal women localized *KISS1* expression to the infundibular nucleus only (Rometo *et al.*, 2007). A more recent study, using both male and female autopsy samples, has confirmed the localization of the majority of kisspeptin cell bodies in the infundibular nucleus, but

identified a second dense population of kisspeptin cells in the rostral preoptic area (Hrabovszky et al., 2010).

Whilst kisspeptin neurones are located in the infundibular/arcuate nucleus across all species, including humans, the rostral population is species specific (Clarkson and Herbison, 2006; Pompolo et al., 2006; Ramaswamy et al., 2008; Clarkson et al., 2009; Hrabovszky et al., 2010). In rodents, the rostral population is located in the anteroventral periventricular nucleus (AVPV) and the periventricular nucleus (PeN), the continuum of this region known as the rostral periventricular region of the third ventricle (RP3V) (Clarkson and Herbison, 2006; Clarkson et al., 2009) (Fig. 1). In contrast, humans and ruminants lack this well-defined RP3V population and have more scattered kisspeptin cell bodies within the preoptic region (Pompolo et al., 2006; Oakley et al., 2009; Hrabovszky et al., 2010). Unlike in humans and ruminants, *Kiss1* mRNA was not detectable in the preoptic area in the adult rhesus monkey (Ramaswamy et al., 2008).

Kisspeptin axons form dense pericapillary plexuses in the human infundibular stalk, the site of neurosecretion of GnRH (Hrabovszky et al., 2010). Axo-somatic, axo-dendritic and axo-axonal contacts between kisspeptin and GnRH axons were also demonstrated in the infundibular stalk, in keeping with data from rodents, sheep and monkeys, where kisspeptin and GnRH neuronal networks are in close proximity (Clarkson and Herbison, 2006; Ramaswamy et al., 2008; Smith et al., 2008a; Hrabovszky et al., 2010; Uenoyama et al., 2011). GnRH neurones express *Kiss1r* mRNA (Irwig et al., 2004; Han et al., 2005; Messager et al., 2005). These findings indicate direct involvement of kisspeptin in the neurosecretion of GnRH. However, in humans as well as other species studied to date, not all GnRH neurones receive kisspeptin neurone contacts and the incidence of these contacts seems low (Clarkson and Herbison, 2006; Ramaswamy et al., 2008; Smith et al., 2008a; Hrabovszky et al., 2010), suggesting a subtle regulation of GnRH secretion by kisspeptin and other neuropeptides.

Kisspeptin neurone populations co-express other neuropeptides

There is considerable overlap in the distribution of kisspeptin, neurokinin B and dynorphin in the hypothalamus, with frequent colocalization. Mapping of kisspeptin and neurokinin B neurones was similar in the infundibular nucleus of post-menopausal women, prompting the identification of a subpopulation of kisspeptin neurones which express neurokinin B and dynorphin in the human infundibular nucleus (Rometo et al., 2007; Hrabovszky et al., 2010). This unique region expressing kisspeptin, neurokinin B and dynorphin is conserved across species and resides in the hypothalamic arcuate nucleus in sheep and rodents (Burke et al., 2006; Goodman et al., 2007; Navarro et al., 2009). Neurokinin B and dynorphin are, however, absent from the kisspeptin population in the preoptic area/RP3V. These infundibular (human)E/arcuate (rodent and ruminant) nucleus neurones which co-express all three neuropeptides are referred to as KNDy neurones (Cheng et al., 2010) (Fig. 1).

KNDy neurones in rats and sheep also co-localize with the glutamate transporter-2, and glutamate has been implicated in mediating estrogen positive feedback resulting in the pre-ovulatory GnRH surge. Whether KNDy cells express glutamate receptors remains to be determined (Pompolo et al., 2003; Ciofi et al., 2006). Kisspeptin neurones in the preoptic area/RP3V are not KNDy neurones, but in the mouse AVPV they co-express tyrosine hydroxylase (the key regulatory step in dopamine synthesis) (Oakley et al., 2009). This differential expression of neuropeptides reflects complex signalling within the hypothalamus and distinct functions of the two kisspeptin populations (Ojeda et al., 2010; Tello et al., 2010).

Kisspeptin neurone populations differ in physiological function

KNDy neurones of the infundibular/arcuate nucleus influence the activity of GnRH by acting on both GnRH cell bodies and neurosecretory terminals (Krajewski et al., 2005; Ciofi et al., 2006; Ramaswamy et al., 2008) (Fig. 1). KNDy neurones make direct contact with GnRH cell bodies and dendrites in humans and project to the median eminence in rodents, sheep and monkeys (Krajewski et al., 2005; Ciofi et al., 2006; Clarkson and Herbison, 2006; Ramaswamy et al., 2008; Dahl et al., 2009). KNDy cells act synergistically to produce coordinated and pulsatile GnRH secretion by controlling the neuroactivity of other KNDy cells, as inferred from a reciprocally interconnected KNDy cell network within the arcuate nucleus in the sheep and rat (Foradori et al., 2002; Burke et al., 2006; Lehman et al., 2010). This is supported by the expression of neurokinin B receptors and the kappa opioid peptide receptor (the receptor for dynorphin) within the KNDy cells, but not the kisspeptin receptor, which predominantly co-localizes with GnRH neurones (Krajewski et al., 2005; Navarro et al., 2009; Herbison et al., 2010) (Fig. 1). This implies that the stimulatory role of neurokinin B and the inhibitory action of dynorphin autosynaptically coordinate the pulsatile release of kisspeptin, which in turn drives the pulsatile secretion of GnRH and LH (Navarro et al., 2009).

Kisspeptin-mediated GnRH stimulation is sex steroid dependent. Estrogen and progesterone modulate kisspeptin activity at both the AVPV nucleus and the arcuate/infundibular nucleus through sex steroid receptors (Ciofi et al., 1994; Goubillon et al., 2000; Foradori et al., 2002; Smith et al., 2005; Franceschini et al., 2006) (Fig. 1). It is becoming clear that not only do kisspeptin neurones mediate both negative and positive sex steroid feedback, but also that distinct subgroups, which are species specific, are involved in these two critical regulatory functions, described more fully below (sections: Kisspeptin mediates negative sex steroid feedback and Kisspeptin may also mediate estrogenic positive feedback). In rodents, the AVPV and the arcuate nucleus respond to positive and negative sex steroid feedback, respectively (Smith et al., 2005, 2006b, Herbison, 2008), whereas in humans, the infundibular nucleus alone relays sex steroid signalling (Rometo et al., 2007; Oakley et al., 2009) (Fig. 1). Thus while there is less marked anatomical differentiation of the two feedback pathways in humans, it remains possible (and perhaps likely) that the two functions are mediated by different neurones.

Kisspeptin neurones show sexual dimorphism

There is evidence for sexual dimorphism in kisspeptin pathways in the human, probably reflecting these functional differences discussed above. Female hypothalami have significantly more kisspeptin fibres in the infundibular nucleus and ventral periventricular zone than are seen in men (Hrabovszky et al., 2010). There is also a striking sex difference in the number and expression of kisspeptin cell bodies, which are observed in the rostral periventricular zone of the female only (Hrabovszky et al., 2010). Likewise only a few kisspeptin cell bodies are present in the infundibular nucleus in males in contrast to the abundant kisspeptin cell bodies in females (Hrabovszky et al., 2010). Similarly, sex differences have been reported in the arcuate nucleus of the sheep (Cheng et al., 2010). Pre-ovulatory positive sex steroid feedback is unique to the female, and the adult female mouse and rat hypothalamus

contain 10-fold more kisspeptin neurones than males in the RP3V region, whereas the arcuate nucleus responsible for negative sex steroid feedback does not display such dimorphism (Clarkson and Herbison, 2006; Kauffman *et al.*, 2007).

Kisspeptin and the regulation of GnRH secretion

Kisspeptin is a potent stimulator of the hypothalamic-pituitary-gonadal (HPG) axis in both animal models and humans. Kisspeptin signals directly to the GnRH neurones through the action on the kisspeptin receptor to release GnRH into the portal circulation, which in turn stimulates the secretion of LH and FSH from the gonadotrophs of the anterior pituitary. Evidence for this comes from multiple sources. Since GnRH cannot be measured in the peripheral circulation, LH pulse frequency remains a widely used and a well validated surrogate of hypophyseal GnRH pulsatility as each GnRH pulse is associated with an LH pulse (Clarke and Cummins, 1985).

Kisspeptin stimulates gonadotrophin release in humans

Kisspeptin stimulates the secretion of both LH and FSH in the human, although the effect on the former is much more marked (George and Seminara, 2012). Kisspeptin-54 was first administered in healthy men by intravenous infusion (4 pmol/kg/min (0.023 μg/kg/min) for 90 min) and resulted in a robust and dose-dependent increase (from 0.25 pmol/kg/min (0.001 μg/kg/min) to 12 pmol/kg/min (0.07 μg/kg/min)) in LH, and less marked rises in FSH and testosterone (Dhillo *et al.*, 2005). Kisspeptin-54 dearance showed first-order kinetics with a half-life of 27.6 ± 1.1 min (Dhillo *et al.*, 2005), which compares with about 4 min for kisspeptin-10 (Jayasena *et al.*, 2011). The potency of kisspeptin to stimulate the secretion of gonadotrophins and its preferential effect on the release of LH has been consistently observed when kisspeptin is administered by different routes (intravenous or subcutaneous) and types of exposure (single boluses or continuous infusion), in different isoforms (kisspeptin-54 and kisspeptin-10), to men or women or in different disease models (Dhillo *et al.*, 2005, 2007; Jayasena *et al.*, 2009, 2010, 2011, 2013a, b; Chan *et al.*, 2011, 2012; George *et al.*, 2011, 2012, 2013; George and Seminara 2012; Abbara *et al.*, 2013; Young *et al.*, 2013). Figures 2 and 3 summarize knowledge of the stimulatory effect of exogenous kisspeptin on the secretion of LH in humans to date.

While kisspeptin stimulates LH release 2- to 3-fold in most circumstances, the stimulatory effect on FSH is much smaller and is less consistent (Dhillo *et al.*, 2005, 2007; George *et al.*, 2011, 2012; Jayasena *et al.*, 2011; Chan *et al.*, 2012). A more potent effect of kisspeptin on LH secretion than FSH in humans is concordant with studies in rodents (Thompson *et al.*, 2004; Navarro *et al.*, 2005a).

Kisspeptin operates upstream of GnRH neurones and coordinates GnRH and LH pulsatility

Kisspeptin acts directly on GnRH neurones

Evidence that kisspeptin exerts its stimulatory function on gonadotrophin secretion through a direct action on the hypothalamic GnRH system is provided by findings from animal studies, consistent with the anatomical studies described above. Kisspeptin causes depolarization of and increases in firing rate of GnRH neurones *in vitro* (Han *et al.*, 2005; Zhang *et al.*, 2008); kisspeptin stimulates the secretion of GnRH in hypothalamic explants (Thompson *et al.*, 2004; Tovar *et al.*, 2006); c-Fos immunoreactivity (a marker of neuronal activity) (Matsui *et al.*, 2004; Han *et al.*, 2005) and the expression of *GnRH* mRNA is up-regulated within the cell bodies of GnRH neurones following the kisspeptin exposure (Novaira *et al.*, 2009; Oakley *et al.*, 2009). In sheep, intracerebroventricular infusion of kisspeptin caused a dramatic increase in the cerebrospinal fluid GnRH content and simultaneously in serum LH and FSH (Messager *et al.*, 2005).

Some studies suggest that kisspeptin directly stimulates pituitary gonadotrophs to release LH and FSH, based on the expression of *Kiss1* and *Kiss1r* genes in gonadotrophs, the secretion of gonadotrophins from pituitary explants treated with kisspeptin (Kotani *et al.*, 2001; Navarro *et al.*, 2005b, Gutierrez-Pascual *et al.*, 2007; Richard *et al.*, 2008) and the detection of kisspeptin (although in low levels) in the hypophyseal portal circulation in the sheep (Smith *et al.*, 2008b). The ability of kisspeptin to induce gonadotrophin release from the pituitary fragments might be explained by the pharmacological concentrations of kisspeptin used (Navarro *et al.*, 2005a, Gutierrez-Pascual *et al.*, 2007). Whilst kisspeptin may have a direct stimulatory action on gonadotrophs, indirect stimulation through enhancing GnRH secretion appears to be the principal physiologic pathway for the stimulation of gonadotrophin secretion (Gottsch *et al.*, 2004; Irwig *et al.*, 2004; Smith *et al.*, 2008b).

The physiological role of kisspeptin in the regulation of GnRH secretion was further demonstrated by studies using a kisspeptin antagonist (Millar *et al.*, 2010). Kisspeptin-induced GnRH neurone firing was abolished by the kisspeptin antagonist (Irwig *et al.*, 2004; Liu *et al.*, 2008; Roseweir *et al.*, 2009). Kisspeptin is needed for the pulsatile release of GnRH, as when injected into the median eminence of pubertal rhesus monkeys, kisspeptin antagonist suppressed both mean GnRH and GnRH pulses (Roseweir *et al.*, 2009). Kisspeptin modulates the secretion of GnRH at the arcuate nucleus, the site of the GnRH pulse generator: kisspeptin antagonist reduced LH pulse frequency when administered to the arcuate nucleus but not when administered to the preoptic area in the rat (Li *et al.*, 2009).

That the effect of kisspeptin on LH release was prevented by pretreatment with GnRH antagonist further points to the action of kisspeptin upstream of GnRH (Gottsch *et al.*, 2004; Shahab *et al.*, 2005). Although there are no studies in humans administering GnRH antagonist followed by kisspeptin, the direct action of kisspeptin on GnRH neurones is inferred from consistent findings in rodents and nonhuman primates. Humans with 'inactivating' mutations in kisspeptin and/or its receptor show hypogonadotropic hypogonadism and delayed puberty (de Roux *et al.*, 2003; Seminara *et al.*, 2003), whist those with 'activating' mutations undergo precocious puberty (Teles *et al.*, 2008; Silveira *et al.*, 2010), suggesting that kisspeptin modulates GnRH pulsatility.

Kisspeptin increases LH pulsatility in humans

As GnRH secretion is pulsatile, the effect of kisspeptin on the characteristics of that pulsatility (as reflected in LH pulses) has been investigated. Intravenous infusion of kisspeptin-10 (1.5 μg/kg/h (1.1 nmol/kg/h) for 9 h) in healthy men (George *et al.*, 2011) and kisspeptin-54 (subcutaneous bolus 0.3 nmol/kg (1.76 μg/kg) and 0.6 nmol/kg (3.5 μg/kg)) in healthy women (Jayasena *et al.*, 2013c) increased LH pulse frequency and amplitude. The ability of kisspeptin to enhance LH pulsatility has also

Subgroup	Isoform	Protocol	LH (IU/L) Baseline	LH (IU/L) Stimulated	Reference
Healthy Men					
	Kp-54	iv 90 min 4pmol/kg/min	4.2	10.8	Dhillo 2005
	Kp-10	iv bolus 0.24nmol/kg	2.8	7.8	Chan 2011
	Kp-10	iv bolus 0.77nmol/kg	4.1	12.4	George 2011
	Kp-10	iv bolus 2.3nmol/kg	4.1	7.0	George 2011
	Kp-10	iv bolus 10nmol/kg	2.9	6.5	Jayasena 2011
	Kp-10	iv 9h 1.1nmol/kg/h	5.2	14.1	George 2011
	Kp-10	iv 22.5h 3.1nmol/kg/h	5.4	20.8	George 2011
Healthy Women					
Follicular	Kp-54	sc bolus 0.4nmol/kg	4.2	4.3	Dhillo 2007
Follicular	Kp-54	sc bolus 0.6nmol/kg	3.4	5.7	Jayasena 2013c
Follicular	Kp-54	sc bolus 6.4nmol/kg	5.7	14.3	Jayasena 2013b
Follicular	Kp-54	sc bolus 6.4nmol/kg BD 5d	9.4	17.7	Jayasena 2013b
Follicular	Kp-54	sc bolus 6.4nmol/kg BD 8d	6.0	18.7	Jayasena 2013b
Follicular	Kp-54	iv bolus 1nmol/kg	3.8	12.1	Jayasena 2011
Follicular	Kp-10	sc bolus 2-32nmol/kg	3.8	3.8	Jayasena 2011
Follicular	Kp-10	iv bolus 0.23nmol/kg	6.3	9.4	George 2012a
Follicular	Kp-10	iv bolus 0.24nmol/kg	2.9	3.8	Chan 2012
Follicular	Kp-10	iv bolus 1-10nmol/kg	3.8	3.8	Jayasena 2011
Follicular	Kp-10	iv 90min 20-720pmol/kg/min	3.8	3.8	Jayasena 2011
Late Follicular	Kp-54	sc bolus 0.4nmol/kg	14.5	35.1	Dhillo 2007
Late Follicular	Kp-10	iv bolus 0.24nmol/kg	34.7	61.3	Chan 2012
Preovulatory	Kp-10	iv bolus 10nmol/kg	6.8	26.8	Jayasena 2011
Luteal	Kp-54	sc bolus 0.4nmol/kg	3.6	5.8	Dhillo 2007
Luteal	Kp-10	iv bolus 0.24nmol/kg	3.4	7.1	Chan 2012
Menopause	Kp-10	iv bolus 0.23nmol/kg	35.3	44.7	George 2012a
Implanon	Kp-10	iv bolus 0.23nmol/kg	4.6	7.5	George 2012a
COCP	Kp-10	iv bolus 0.23nmol/kg	2.3	3.7	George 2012a

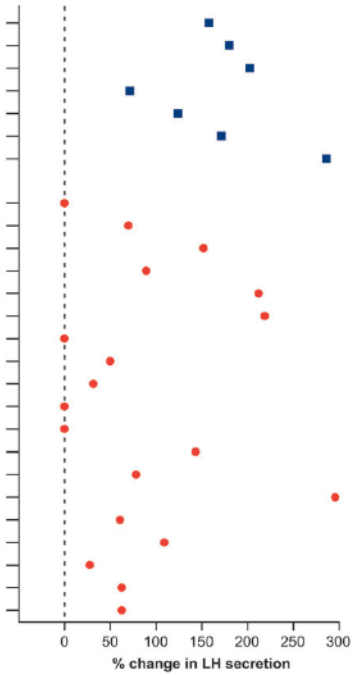

Figure 2 Kisspeptin stimulates LH secretion in healthy men (filled squares) and women (filled circles). The stimulatory effect of kisspeptin on LH secretion is shown in both healthy men and women, when kisspeptin is administered in different isoforms (kisspeptin-54 and kisspeptin-10) and by different protocols (intravenous or subcutaneous, single boluses or continuous infusion). Note that stimulated LH values are either mean LH or peak LH concentrations depending on how the data are originally presented. Where authors do not state exact LH concentration following kisspeptin administration, this was obtained from the relevant figures. 0% change in LH secretion is reported if no statistically significant change in LH secretion was reported and authors do not show actual LH concentrations. iv, intravenous; sc, subcutaneous; BD, twice daily; Implanon, etonogestrel contraceptive implant; COCP, combined oral contraceptive pill.

been demonstrated in human reproductive disorders, including in hypothalamic amenorrhoea (Jayasena *et al.*, 2013a), in hypogonadal men with type 2 diabetes (George *et al.*, 2013) and in neurokinin B signalling defects (Young *et al.*, 2013), described more fully below. Kisspeptin not only drives the pulsatile secretion of GnRH, but also appears to reset the hypothalamic clock of GnRH pulsatility in men. Acute injection of kisspeptin-10 delayed the next endogenous LH pulse by the interval that would be observed between the two consecutive endogenous LH pulses (Chan *et al.*, 2011). However, the same kisspeptin dosing protocol did not support the ability of kisspeptin to reset the GnRH pulse generator in women across the different phases of the menstrual cycle (Chan *et al.*, 2012). The authors suggest that the GnRH pulse generator in men operates differently to women and that it is the changes in the sex steroid milieu across the menstrual cycle in women that might be responsible for this discrepancy (Chan *et al.*, 2012). The marked sexual dimorphism in the anatomy of the kisspeptin system described above may underlie this intriguing observation and even determine the frequency of GnRH secretion in women across the normal menstrual cycle. This variability in the frequency of GnRH pulsatility is central to the differential regulation of LH and FSH (McNeilly *et al.*, 2003) and thus ovarian follicle development, the correct selection of a single dominant follicle for ovulation, and the luteal phase with limited follicle development.

Men and women show sexual dimorphism in their response to kisspeptin

Men and women display sexual dimorphism in their response to exogenous kisspeptin (Fig. 2). Whilst kisspeptin potently stimulates the release of LH in men, the effect of kisspeptin is more variable in women and depends on the phase of the menstrual cycle. It has been proposed

that in the early follicular phase, the impact of exogenous kisspeptin is limited due to high endogenous kisspeptin activity (Chan *et al.*, 2012), although this is speculative. Sex steroid-deficient post-menopausal women were more responsive to kisspeptin-10 than women in the early follicular phase (George *et al.*, 2012) (Fig. 2). Women taking combined estrogen and progestogen contraceptives showed a minimal response to kisspeptin-10 (George *et al.*, 2012), contrasting to the larger response in the physiological luteal phase (Dhillo *et al.*, 2007) (Fig. 2). These complex relationships suggest that other mechanisms, in addition to the absolute or relative levels of estrogen and progesterone, appear to regulate kisspeptin sensitivity across the menstrual cycle and clearly there remains much to be learnt regarding these inter-relationships. The expression of kisspeptin receptor in different sex steroid environments has not been described in primates and data in lower species remain contradictory (Navarro *et al.*, 2004a, Yamada *et al.*, 2007; Li *et al.*, 2012). Changes in pituitary sensitivity to GnRH and sex steroid feedback at that level (Hall *et al.*, 1994; Shaw *et al.*, 2010) add to the complexity of analysis of *in vivo* studies.

The sexual dimorphism in the responsiveness of men and women has been elegantly illustrated using the different isoforms of kisspeptin (Jayasena *et al.*, 2011). Men respond to modest doses of both kisspeptin-54 and kisspeptin-10. In a study of healthy women in the early follicular phase, kisspeptin-10 administered as an intravenous bolus, subcutaneous bolus or as an intravenous infusion did not result in a detectable LH response (Jayasena *et al.*, 2011) (Fig. 2). In another study however, a low-dose intravenous bolus of kisspeptin-10 induced an LH response in normal women in the early follicular phase (George *et al.*, 2012). These differences may have been methodological—as the Jayasena *et al.* (2011) protocol did not involve baseline sampling. In the study by George *et al.* (2012), a 10-min baseline LH sampling for 180 min was employed prior to kisspeptin administration, enabling comparison of LH secretion before and after kisspeptin-10 infusion in the same individual. Given the small sample number, small effect size and inter-individual variability in baseline LH, this lack of baseline data to enable intra-individual comparisons diminishes statistical sensitivity to identify small changes in LH. A small but significant increase in LH in response to kisspeptin-54 administered intravenously or subcutaneously in the early follicular phase was also observed (Dhillo *et al.*, 2007; Jayasena *et al.*, 2011), indicating that the response to the longer isoform is substantially more robust, perhaps reflecting its longer half-life. Sexual dimorphism in the kisspeptin system is also seen in rodent models: females have significantly more kisspeptin neurones than males in the AVPV nucleus (Oakley *et al.*, 2009). This sexual variation in anatomical distribution of the kisspeptin pathway and the response to kisspeptin administration may reflect sexually dimorphic roles of kisspeptin, notably in the generation of the pre-ovulatory LH surge, which is unique to the female.

Kisspeptin mediates negative sex steroid feedback

Patterns of GnRH and subsequently LH secretion across the menstrual cycle are modulated by gonadal steroid feedback. During the follicular phase of the menstrual cycle GnRH activity and thus LH secretion is limited by estradiol (E_2)-mediated negative feedback (with additional action on pituitary gonadotrophs) (Karsch, 1987; Shaw *et al.*, 2010). The basis for the change to positive feedback with elaboration of the mid-cycle LH surge has long been unclear. GnRH neurones do not express ERs, suggesting that a separate population of neurones acts as a mediator to relay the ovulation-inducing message from gonads to the hypothalamic GnRH neurones. Recent evidence suggests that KNDy neurones appear to constitute this 'missing link', mediating both negative and positive sex steroid feedback.

Kisspeptin in the infundibular nucleus mediates negative feedback of estrogen in humans (Fig. 1). In post-menopausal women kisspeptin neurones in the infundibular nucleus were hypertrophied and expressed more *KISS1* mRNA than in premenopausal women (Rometo *et al.*, 2007). These hypertrophied neurones expressed both *ESR1* (encoding ER alpha) and neurokinin B mRNA, had increased expression of neurokinin B and showed a similar distribution to that of kisspeptin neurones (Rance *et al.*, 1990; Rance and Young, 1991). The suggestion that kisspeptin and neurokinin B in the infundibular nucleus act synergistically to mediate estrogen negative feedback is supported by animal data, showing an up-regulation of *Kiss1* mRNA expression in ovariectomised rodents, sheep and monkeys in the arcuate nucleus (equivalent to the infundibular nucleus in humans) but not in more rostral areas, and that this was prevented by E_2 replacement (Oakley *et al.*, 2009; Lehman *et al.*, 2010). Consistent with this, the intracerebroventricular administration of kisspeptin antagonist prevented the LH rise in castrated rodents (Roseweir *et al.*, 2009). Similarly, ovariectomy increased and estrogen replacement reduced neurokinin B gene expression in the infundibular nucleus of monkeys (Abel *et al.*, 1999; Sandoval-Guzman *et al.*, 2004).

These findings together imply that estrogen mediates its negative feedback by suppressing kisspeptin and neurokinin B release from KNDy neurones, which reduces their stimulatory input to GnRH neurones (Fig. 1). The converse, i.e. inhibitory, involvement of the opioid component of this signalling system has long been recognized. The colocalization of dynorphin in at least some kisspeptin and neurokinin B-containing neurones in the human is discussed above (section: Kisspeptin neurone populations co-express other neuropeptides). Naloxone, an opioid receptor antagonist, increased serum LH levels in late follicular and luteal phases of the menstrual cycle (Quigley and Yen, 1980; Shoupe *et al.*, 1985). This effect was not apparent in post-menopausal and oophorectomized young women, whereas replacement of estrogen or progesterone restored the ability of naloxone to release LH (Melis *et al.*, 1984; Casper and Alapin-Rubillovitz, 1985; Shoupe *et al.*, 1985). The endogenous opioid peptide dynorphin mediates this role physiologically, and inhibited GnRH and LH pulse frequency following progesterone administration (Ferin *et al.*, 1984; Karsch, 1987; Goodman *et al.*, 2004). In contrast, the opioid receptor antagonist naltrexone increased serum LH concentrations and LH pulse amplitude in women with hypothalamic amenorrhoea (Genazzani *et al.*, 1995). The relative deficiency of dynorphin signalling as part of negative estrogen feedback in the post-menopausal and oophorectomized states would appear a likely explanation for the lack of response to naloxone in hypergonadotrophic states, in contrast to potentially increased dynorphin signalling contributing to the hypogonadotrophic state in hypothalamic amenorrhoea. It is however possible that already near maximal LH secretion in sex steroid deficient post-menopausal and oophorectomized women would account for the inability of naloxone to further stimulate gonadotrophin release although kisspeptin-10 induced LH secretion in post-menopausal women (George *et al.*, 2012). In post-menopausal women, the expression of prodynorphin mRNA in the infundibular nucleus is reduced (Rometo and Rance, 2008). The role of dynorphin as a mediator of sex steroid negative feedback is consistent with data

from the ewe, where dynorphin is coexpressed with kisspeptin and neurokinin B, both of which show a high degree of colocalization with ER alpha and progesterone receptors in the arcuate nucleus, and the expression of prodynorphin mRNA is suppressed by ovariectomy (Goubillon *et al.*, 2000; Foradori *et al.*, 2002, 2005; Franceschini *et al.*, 2006; Goodman *et al.*, 2007). This is distinct from the apparent situation in rodents where, despite colocalization of KNDy neurones with both estrogen and progesterone receptors, dynorphin does not seem to mediate estrogen negative feedback (Navarro *et al.*, 2009).

In summary, it appears that in humans KNDy neurones mediate negative sex steroid feedback in the infundibular nucleus by suppressing the secretion of kisspeptin and neurokinin B and stimulating the secretion of dynorphin, which act synergistically to reduce the activity of the GnRH neuronal system, and thus gonadotrophin secretion.

Kisspeptin may also mediate estrogenic positive feedback

Estrogen feedback switches from negative to positive in the late follicular phase to induce the GnRH/LH surge at the time of ovulation. However, the neuroendocrine mechanisms involved in this critical physiological event have been unclear. Emerging data suggest that although the negative feedback of sex steroids is mediated by KNDy neurones in the infundibular/arcuate nucleus, the positive feedback of sex steroids is more site and species specific (Fig. 1).

Recent data support a potential role for kisspeptin in generating the ovulatory LH surge in humans. Kisspeptin-54 (subcutaneous in doses of 1.6–12.8 nmol/kg (9.4–75 μg/kg), used instead of hCG during an FSH/GnRH antagonist assisted conception ovulation induction protocol, induced an LH surge and triggered oocyte maturation, with subsequently a live term birth reported (Abbara *et al.*, 2013). Repeated twice-daily administration of kisspeptin-54 shortened the menstrual cycle and advanced the onset of the LH peak in healthy women (Jayasena *et al.*, 2013b). This is in keeping with data from animal models. Kisspeptin administration results in an early LH surge in sheep, and conversely administration of kisspeptin antiserum or antagonists to rats and sheep prevented or blunted the LH surge (Kinoshita *et al.*, 2005; Caraty *et al.*, 2007; Clarkson *et al.*, 2008; Pineda *et al.*, 2010).

The anatomical site of kisspeptin that relays positive sex steroid feedback is different in rodents compared with humans and other species. In rodents the AVPV nucleus is the location of estrogen positive feedback, which is not matched in humans, other primates and sheep, where kisspeptin neurones in the infundibular/arcuate exert this function (Fig. 1). The expression of *Kiss1* mRNA in the AVPV nucleus is dramatically increased after estrogen replacement and at the time of the GnRH/LH surge (Smith *et al.*, 2005, 2006b). There are no studies looking at the anatomical region of kisspeptin expression that mediates estrogen positive feedback in humans and evidence comes from other species, which, like humans, have no homologous area to the AVPV nucleus. In sheep, the expression of *Kiss1* mRNA in the arcuate nucleus is markedly enhanced during the pre-ovulatory LH surge (Smith *et al.*, 2008b). In rodents the AVPV nucleus receives afferent fibres from the suprachiasmatic nucleus, the location of circadian clock, which coordinates and provides precise timing for the LH surge and the kisspeptin system in now being integrated into our understanding of the neurobiology of this system across species, including the human (Christian and Moenter, 2010; Khan and Kauffman, 2012).

KNDy neurones may have a role in positive estrogen feedback. In sheep, the neurokinin B receptor agonist senktide increased LH secretion close to levels seen during the pre-ovulatory LH surge (Billings *et al.*, 2010). KNDy neurones do not mediate positive estrogen feedback in rodents based on their location in the arcuate nucleus only (Burke *et al.*, 2006; Goodman *et al.*, 2007; Navarro *et al.*, 2009). Other neurotransmitters may also contribute to the kisspeptin-mediated LH surge, as kisspeptin populations in the preoptic area/RP3V and the infundibular/arcuate nucleus co-localize with different peptides (see above section: Kisspeptin neurone populations co-express different neuropeptides).

Kisspeptin stimulates gonadotrophin release in disease models

In addition to being a potent stimulator of LH secretion in healthy men and women, the ability of kisspeptin to induce LH release in human disease models characterized by low gonadotrophin secretion has been investigated (Fig. 3).

Hypothalamic amenorrhoea

Hypothalamic amenorrhoea is characterized by slow GnRH pulsatile secretion, resulting in a preferential decline in LH compared with FSH secretion and low ovarian follicular activity. As, by definition, this is a functional rather than pathological condition, it might be readily corrected by administration of kisspeptin to increase GnRH secretion. Hypothalamic amenorrhoea was the first disease model used to explore the therapeutic potential of kisspeptin-54, which when administrated as subcutaneous bolus at 6.4 nmol/kg (37 μg/kg) resulted in a 10-fold increase in LH and 2.5-fold increase in FSH secretion, both to normal physiological levels (Jayasena *et al.*, 2009) (Fig. 3). However, this increase in gonadotrophins did not translate into a significant elevation in E_2 secretion, suggesting that folliculogenesis was not restored, confirmed by ovarian quiescence on ultrasound scan (Jayasena *et al.*, 2009). The lack of ovarian activity may relate to the limited effect on FSH secretion and the short timescale of study. Despite the initial stimulation of LH and FSH secretion, when kisspeptin-54 was injected twice daily for 2 weeks, these increases were not sustained with LH falling to pretreatment levels, suggesting tachyphylaxis (Jayasena *et al.*, 2009, 2010) (see section: Continuous exposure to kisspeptin can cause desensitization). However, sustained secretion of gonadotrophins at physiological levels was achieved with intermittent subcutaneous injection of kisspeptin-54 twice weekly (6.4 nmol/kg (37 μg/kg)) for 8 weeks, although it did not result in increased E_2 secretion or follicular development (Jayasena *et al.*, 2010). It has subsequently been shown that an infusion of kisspeptin-54 (0.01 nmol/kg/h (0.059 μg/kg/h) to 1 nmol/kg/h (5.9 μg/kg/h)) for 8 h in women with hypothalamic amenorrhoea can induce LH pulsatility with a 3-fold increase in LH pulse frequency and mass per pulse (Jayasena *et al.*, 2013a). The ability of the increased gonadotrophin secretion, and perhaps the relative effects of LH and FSH, to bring about ovarian activity and menstrual cycles will determine the therapeutic application of kisspeptin in this condition.

In all the studies above, regardless of the dose and route of administration, the LH response to kisspeptin is ~5-fold greater in hypothalamic amenorrhoea than in healthy women in the early follicular phase. This is consistent with data from a rodent model of undernutrition showing up-regulated hypothalamic *Kiss1r* mRNA expression (Castellano *et al.*, 2005). Altered GnRH sensitivity is unlikely as the effect of GnRH on

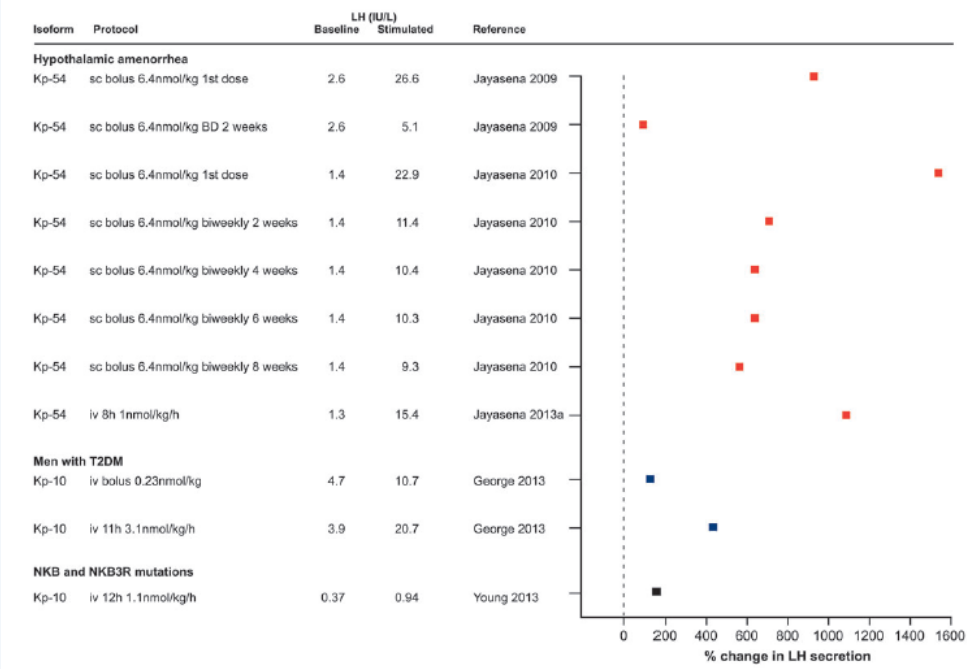

Figure 3 Increase in LH secretion following the administration of kisspeptin in human disease models. The stimulatory effect of kisspeptin on LH secretion is shown in reproductive endocrine disorders, when kisspeptin is administered in different isoforms (kisspeptin-54 and kisspeptin-10) and by different protocols (i.v. or s.c., single boluses or continuous infusion) as indicated. Note that stimulated LH values are either mean LH or peak LH concentrations depending on how the data are originally presented. Where authors do not state an exact LH concentration following kisspeptin administration, this is obtained from the relevant figures. BD, twice daily; T2DM, type 2 diabetes; NKB *(TAC3)*, neurokinin B; NKB3R *(TAC3R)*, neurokinin B receptor.

LH secretion is similar in hypothalamic amenorrhoea and healthy women in the early follicular phase (Jayasena *et al.*, 2009; George *et al.*, 2012).

Hypogonadism in men with type 2 diabetes

Men with type 2 diabetes often have low testosterone concentrations, and inappropriately low LH indicating a hypothalamic/pituitary basis (George *et al.*, 2010). As with hypothalamic amenorrhoea, increasing LH secretion by administration of kisspeptin might therefore have therapeutic potential. This has been explored in a small number of such men, investigating the response to both bolus administration and infusion of kisspeptin-10 (George *et al.*, 2013) (Fig. 3). Kisspeptin-10 intravenous bolus administration (0.3 μg/kg (0.23 nmol/kg)) increased LH secretion 2-fold in diabetic hypogonadal men, i.e. of the same magnitude as in healthy men with peak mean LH 10.7 IU/l and 14.5 IU/l, respectively (George *et al.*, 2013). An infusion of kisspeptin-10 for 11 h at a higher dose (4 μg/kg/h (3.1 nmol/kg/h)) produced a more profound 5-fold increase in LH release (George *et al.*, 2013), also comparable to the response in healthy men (George *et al.*, 2011). Kisspeptin-10 also stimulated LH pulse frequency in diabetic men with hypogonadotropic hypogonadism, which was sustained for the duration of the infusion (11 h) with no evidence of a decline in LH (i.e. no desensitization) over that timescale (George *et al.*, 2013). Importantly, serum testosterone was also elevated into the normal physiological range (George *et al.*, 2013). The ability of kisspeptin to robustly increase LH pulsatility with an associated increase in testosterone is very encouraging, but the potential of kisspeptin to maintain gonadotrophin and sex steroid release for longer periods of time relevant to therapeutic use has yet to be determined.

Neurokinin B signalling deficiencies

Patients with loss-of-function mutation in neurokinin B (*TAC3*) and its receptor (*TAC3R*) show hypogonadotropic hypogonadism characterized by failure to progress through puberty (Topaloglu *et al.*, 2009). It is postulated that inability of neurokinin B in an autocrine and/or paracrine manner to stimulate kisspeptin secretion results in low GnRH pulse frequency with correspondingly low LH and gonadal steroid levels but normal or near-normal levels of FSH typically seen in these patients. Neurokinin B, being potentially upstream of kisspeptin in neuroendocrine

signalling, makes kisspeptin an attractive therapeutic agent to restore GnRH secretion in patients with defects in the neurokinin B system. Indeed, continuous infusion of kisspeptin-10 (1.5 μg/kg/h (1.1 nmol/kg/h) for 12 h) in two patients with *TAC3* and two patients with *TAC3R* mutation stimulated the LH response 2.5-fold (Young *et al.*, 2013) (Fig. 3). Overall, the LH response to kisspeptin was more limited than that achieved in healthy men using the same protocol (George *et al.*, 2011) with lower LH mass per pulse, although pulse frequency was normalized, consistent with complex neuropeptide interactions associated with KNDy neurone function rather than a linear hierarchy, as described above. Nevertheless, a significant increase in testosterone levels in male patients and in E_2 levels in the single female patient was achieved (Young *et al.*, 2013).

Continuous exposure to kisspeptin can cause desensitization

Continuing administration of GnRH desensitizes the HPG axis after an initial stimulation, by down-regulation of GnRH receptors and desensitization of gonadotrophes (Belchetz *et al.*, 1978; McArdle *et al.*, 1987; Mason *et al.*, 1994). There is evidence for pulsatile (i.e. non-continuous) secretion of kisspeptin within the hypothalamic median eminence of the monkey (Keen *et al.*, 2008). Continuous administration of kisspeptin-10 (intravenous 200 μg/h (154 nmol/kg) or 400 μg/h (307 nmol/kg) for 98 h) to rhesus monkeys resulted in suppressed LH secretion, indicative of kisspeptin receptor desensitization (Ramaswamy *et al.*, 2007). The kisspeptin receptor has also been shown to desensitize *in vitro* (Pampillo *et al.*, 2009). Consistent with this, repeated subcutaneous administration of kisspeptin-54 (6.4 nmol/kg (37 μg/kg) twice daily) for 2 weeks in women with hypothalamic amenorrhoea resulted in an initial stimulation of LH and FSH which was not maintained (Jayasena *et al.*, 2009) (Fig. 3). However other studies in humans using infusions or repeated administration of kisspeptin have not provided consistent evidence for desensitization (Figs 2 and 3). More recently, infusion of a lower dose of kisspeptin-54 for 8 h (from 0.01 nmol/kg/h (0.059 μg/kg/h) to 1 nmol/kg/h (5.9 μg/kg/h)) in women with hypothalamic amenorrhoea not only caused sustained LH secretion but also restored LH pulsatility (Jayasena *et al.*, 2013a) (Fig. 3). Continuous exposure to kisspeptin-54 administered twice daily for 1 week advanced the menstrual cycle in healthy women (Jayasena *et al.*, 2013b). Similarly, continuous kisspeptin-10 infusion at 4 μg/kg/h (3.1 nmol/kg/h) for 22.5 h in healthy men showed continuing stimulation of LH secretion, with no evidence of desensitization (George *et al.*, 2011) (Fig. 2). In contrast, LH secretion was not sustained in three healthy men during infusion of kisspeptin 10 for 24 h at 12 μg/kg/h (9.2 nmol/kg/h), the highest dose used in humans to date (Lippincott *et al.*, 2013). However, LH secretion remained well above baseline at the end of infusion, in contrast to the marked desensitization observed with a high dose of kisspeptin-54 in women with hypothalamic amenorrhoea (Jayasena *et al.*, 2009) (Fig. 2). It would be interesting to determine if LH secretion remains above baseline or if LH decreases to castrate levels with kisspeptin infusion for longer than 24 h. These data therefore suggest that while high doses of both kisspeptin-54 and kisspeptin-10 may induce desensitization, this does not occur at lower doses. In a dose-finding study involving bolus injection of kisspeptin-10, the highest dose (3 μg/kg (2.3 nmol/kg)) resulted in a sub-maximal LH response, consistent with desensitization even with bolus administration of kisspeptin-10 (George *et al.*, 2011) (Fig. 2). An alternative explanation for this observation is that at that high dose kisspeptin might have stimulated another RF-amine receptor, such as gonadotrophin inhibitory hormone receptor, known to have an inhibitory effect on GnRH and LH (George *et al.*, 2011).

Consistent with these findings, intermittent administration of kisspeptin results in sustained GnRH and LH pulsatility. Intermittent administration of kisspeptin-10 in juvenile male monkeys (intravenous hourly for 2 days) and juvenile female rats (intracerebroventricular twice daily for 5 days) caused precocious puberty (Navarro *et al.*, 2004b, Plant *et al.*, 2006). Kisspeptin-54 (6.4 nmol/kg (37 μg/kg/h)) injected twice weekly sustained the secretion of gonadotrophins for an 8-week period after a brief initial suppression (Jayasena *et al.*, 2010) (Fig. 3).

The ability of natural forms of kisspeptin to induce desensitization in humans thus remains unclear, with the discrepancies between studies possibly reflecting the duration of kisspeptin injection (8–22.5 h versus 2 weeks), lower doses of kisspeptin infused in the human studies compared with primate studies, variation in the isoforms of kisspeptin used, the mode of kisspeptin administration, differences between the human and animal models and sex and even health status (healthy men versus women with hypothalamic amenorrhoea). Kisspeptin receptor agonist analogues have also been developed, and two of these, TAK-448 and TAK-683, are potent inducers of desensitization with potential use to suppress gonadotrophin secretion and thus gonadal function, similar to the GnRH analogues widely used today. Phase I clinical studies in healthy men show that subcutaneous infusion of TAK-448 (0.01–1 mg/day) and TAK-683 (0.01–2 mg/day) for 2 weeks rapidly suppress testosterone below castration levels (MacLean *et al.*, 2013; Scott *et al.*, 2013).

Kisspeptin and puberty

The demonstration of the obligate role of kisspeptin-GPR54 signalling in human puberty was the finding that firmly established kisspeptin as a crucial regulator of reproductive function. In 2003, two independent groups identified 'inactivating' point mutations and deletions in *KISS1R* that were associated with impaired pubertal development in some patients with hypogonadotropic hypogonadism (de Roux *et al.*, 2003; Seminara *et al.*, 2003). Mutations in *KISS1R* were demonstrated in both consanguineous families and in unrelated patients. In addition, Kiss1r- and Kiss1-deficient mice displayed a virtually identical phenotype (Funes *et al.*, 2003; Seminara *et al.*, 2003; d'Anglemont de Tassigny *et al.*, 2007).

Conversely an 'activating' mutation (Arg386Pro) in the kisspeptin receptor gene was identified in a girl with precocious puberty, although inheritance could not be determined as the biological family was not available for genetic testing (Teles *et al.*, 2008). Cells transfected with the mutant kisspeptin receptor showed prolonged accumulation of inositol phosphate and phosphorylation of extracellular signal-regulated kinase, indicating extended intracellular signalling (Teles *et al.*, 2008). Missense mutations have also been described in the *KISS1* gene in three unrelated children with central precocious puberty (Silveira *et al.*, 2010). This mutant kisspeptin is more resistant to *in vitro* degradation, suggesting greater bioavailability as the cause of precocious puberty (Silveira *et al.*, 2010).

Hypothalamic expression of *Kiss1 and Kiss1r* mRNA is dramatically up-regulated at puberty in rodents and primates (Navarro *et al.*, 2004a, Han *et al.*, 2005; Shahab *et al.*, 2005) and the percentage of GnRH neurones depolarizing in response to kisspeptin increases from

juvenile (25%) to prepubertal (50%) to adult mice (>90%), suggesting that GnRH neurones acquire sensitivity to kisspeptin across puberty (Han *et al.*, 2005). Kisspeptin-54 secretion and specifically kisspeptin-54 pulse frequency increase at the onset of puberty in monkeys (Keen *et al.*, 2008). In addition to these physiological changes linking kisspeptin signalling to the timing of puberty, the administration of exogenous kisspeptin resulted in earlier puberty in rats and monkeys (Navarro *et al.*, 2004b, Plant *et al.*, 2006). Conversely, administration of a kisspeptin antagonist inhibited pulsatile GnRH release in pubertal monkeys and delayed puberty in rats (Roseweir *et al.*, 2009; Pineda *et al.*, 2010). The findings strongly support a requirement for KISS1/GPR54 signalling to initiate and progress through puberty in a range of species.

Kisspeptin and metabolism

Human reproductive function is influenced by both extremes of nutrition—undernutrition and obesity. Kisspeptin may provide a link between nutritional/metabolic status and reproduction by sensing energy stores and translating this information into the pulsatile secretion of GnRH. The expression of *Kiss1* mRNA and gonadotrophin secretion is reduced in mice, pubertal rats and monkeys subject to fasting (Castellano *et al.*, 2005; Cota *et al.*, 2006; Roa *et al.*, 2009; Wahab *et al.*, 2011). Kisspeptin is able to restore delayed vaginal opening and increases low gonadotrophin and estrogen levels associated with chronic undernutrition in pre-pubertal rats (Navarro *et al.*, 2004b, Castellano *et al.*, 2005).

Humans with mutations in leptin or leptin receptor show hypogonadism (Farooqi and O'Rahilly, 2009). The leptin receptor (Ob-Rb) is not present on GnRH neurones, but 40% of kisspeptin neurones in the mouse arcuate nucleus express the leptin receptor (Smith *et al.*, 2006a), suggesting a role for kisspeptin in mediating the metabolic signals of leptin on the HPG axis. Leptin-deficient mice show decreased expression of *Kiss1* mRNA, which is partially up-regulated by leptin (Smith *et al.*, 2006a). Incomplete restoration of *Kiss1* mRNA expression suggests that other mediators are involved in inhibiting kisspeptin signalling in leptin deficiency. Furthermore, mice with selective deletion of leptin receptor from kisspeptin neurones display normal pubertal development, sexual maturation and fertility, demonstrating that leptin action on kisspeptin neurones is not obligatory for these processes (Donato *et al.*, 2011).

Low levels of testosterone have also been observed in men with obesity and type 2 diabetes, where decreased secretion of GnRH is thought to be the causative factor (Dandona *et al.*, 2008). A rat model of diabetes (streptozocin treated) has reduced levels of hypothalamic *Kiss1* mRNA with subsequently low levels of circulating gonadotrophins and sex steroids, which are corrected by kisspeptin (Castellano *et al.*, 2006, 2009). This raises the possibility that diminished kisspeptin secretion is a potential mechanism for hypogonadotropic hypogonadism in patients with obesity and diabetes (George *et al.*, 2010). Indeed, as described above, kisspeptin-10 increased LH pulse frequency and LH secretion in hypogonadal men with type 2 diabetes (George *et al.*, 2013). The likely pathways for down-regulation of kisspeptin signalling include negative feedback by estrogen, which is markedly elevated in obesity (Schneider *et al.*, 1979), resistance to leptin, also seen in human obesity (Finn *et al.*, 1998), insulin resistance and hyperglycaemia (Castellano *et al.*, 2006, 2009), and inflammation, which is up-regulated in hypogonadal men with diabetes (Dandona *et al.*, 2008) and is associated with decreased kisspeptin expression in rats (Iwasa *et al.*, 2008).

Current data indicate that kisspeptin acts downstream to metabolic signals and conveys information about energy stores to GnRH neurones, thereby regulating reproduction. This gives promise for a potential novel therapeutic role of kisspeptin to restore the reproductive axis in conditions of negative energy balance, such as anorexia nervosa, and in diabetes.

Clinical applications of KNDy manipulation

GnRH analogues are extensively used in clinical practice in the treatment of hormone-dependent diseases and infertility. Current therapies manipulate the HPG axis at the level of GnRH receptors on pituitary gonadotrophs, largely to suppress gonadal function, e.g. in the treatment of prostate and breast cancer, endometriosis and uterine fibroids. As reproductive endocrine conditions can be broadly categorized into those with pathologically diminished (delayed puberty, hypothalamic amenorrhoea, hypogonadism in diabetes) and pathologically enhanced (polycystic ovary syndrome (PCOS), menopause, precocious puberty) GnRH and associated gonadotrophin pulsatility, the newly discovered hypothalamic peptides kisspeptin and neurokinin B offer a novel therapeutic approach with potential advantages over the existing therapies in several clinical contexts (Fig. 4).

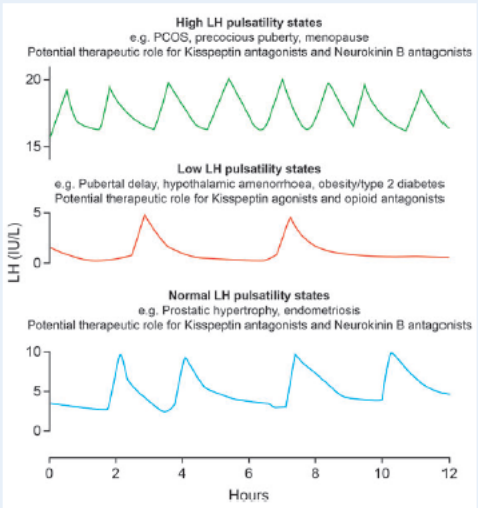

Figure 4 Potential clinical applications of novel kisspeptin-based modulation of LH secretion. Schematic presentation of LH pulses in health and in reproductive endocrine disorders. In health, an LH pulse occurs about every 90 min. The frequency of LH pulses is diminished in patients with hypothalamic amenorrhoea, male hypogonadism and pubertal delay, whereas LH pulse frequency is enhanced in women with polycystic ovary syndrome, menopause and precocious puberty. Therapeutic opportunities to correct abnormal LH pulse frequency by manipulating KNDy neurones with relevant agonists and antagonists are emerging. PCOS, polycystic ovary syndrome.

Manipulation of KNDy neurones to stimulate HPG axis

Enhancing the stimulatory tone of kisspeptin and neurokinin B by appropriate agonists and suppressing the inhibitory tone of dynorphin by its antagonists, may have therapeutic potential for diseases with decreased gonadotrophin secretion. Exogenous kisspeptin enhances diminished LH pulsatility in hypogonadal men with diabetes and stimulates LH secretion in women with hypothalamic amenorrhoea (Jayasena *et al.*, 2010; George *et al.*, 2013). Kisspeptin initiates puberty in monkeys and rodents, but this has not been tested in children with delayed puberty (Navarro *et al.*, 2004b, Plant *et al.*, 2006). Pulsatile gonadotrophin secretion is restored by kisspeptin administered to patients with hypogonadotropic hypogonadism secondary to mutations in neurokinin B and/or its receptor (Young *et al.*, 2013). The role of dynorphin antagonists, such as naloxone, in patients with abnormally low LH secretion remains to be elucidated.

Kisspeptin therapy has the potential to 'fine tune' IVF techniques. Kisspeptin triggered the LH surge during following ovulation induction for assisted reproduction (Abbara *et al.*, 2013) with successful achievement of a live birth. Kisspeptin might stimulate a more physiological pattern of gonadotrophin secretion, avoiding the risk of ovarian hyperstimulation syndrome associated with currently used hCG injections although clearly much remains to be discovered regarding potential advantages and disadvantages over current approaches.

Manipulation of KNDy neurones to inhibit HPG axis

Suppressing the stimulatory role of kisspeptin and neurokinin B by specific receptor antagonists and enhancing the inhibitory action of dynorphin by its receptor agonist is desirable in scenarios of increased GnRH pulsatility where a reduction rather than complete suppression of GnRH is required. Increased frequency of GnRH and therefore LH pulsatile secretion (with little effect on FSH secretion) is central to the pathophysiology of PCOS, the most common endocrinopathy in women. As GnRH pulse frequency primarily determines LH but not FSH secretion (McNeilly *et al.*, 2003), slowing GnRH might normalize the relative LH hypersecretion often seen in PCOS. Normalization of LH secretion (and perhaps the consequent hyperandrogenism) in PCOS may promote folliculogenesis and ovulation. Studies using a neurokinin B antagonist are currently underway to reduce high LH secretion in PCOS.

The ability of kisspeptin antagonists to limit follicular development and inhibit ovulation offers potential for a novel female contraceptive, perhaps being specifically advantageous in the scenarios where exogenous estrogen is contraindicated. However it might be limited by the resulting lack of progesterone exposure and adverse effects on the endometrium. Given the preferential stimulation of LH secretion in response to kisspeptin in humans (Dhillo *et al.*, 2005, 2007; George *et al.*, 2011, 2012), kisspeptin antagonists might potentially result in relative sparing of FSH compared with LH, which might reduce or prevent the unwanted side effects of estrogen deficiency, such as vasomotor symptoms and risk of osteoporosis, associated with GnRH analogue administration.

A similar therapeutic approach might support the use of kisspeptin and neurokinin B suppressive therapies in the treatment of precocious puberty. Similarly, it may alleviate menopausal hot flushes since KNDy neurones project to preoptic thermoregulatory areas that express neurokinin B receptor in rats and KNDy neurone ablation reduces cutaneous vasodilation (Burke *et al.*, 2006; Rance, 2009; Hrabovszky *et al.*, 2010; Krajewski *et al.*, 2010; Mittelman-Smith *et al.*, 2012; Rance *et al.*, 2013). Although the inhibitory role of opioids on GnRH and LH pulsatility is well known, manipulation of this system does not have the apparent specificity of the kisspeptin or neurokinin B pathways.

The potential more subtle effects of kisspeptin antagonists reducing LH pulsatility contrast with the profound suppression resulting from GnRH analogue administration, decreasing gonadotrophin and sex steroid secretion to castration levels with consequent side effects, including hot flushes, loss of libido and decreased bone mineral density (Roseweir *et al.*, 2009). Complete suppression of gonadotrophins and sex steroids is necessary is some conditions, such as prostate cancer, but partial suppression is more appropriate in benign prostatic hyperplasia, endometriosis and uterine fibroids. Clinical effectiveness in the management of endometriosis and uterine fibroids with GnRH suppression with add back, and with selective progesterone receptor modulators (Chabbert-Buffet *et al.*, 2005), suggests that approaches not based on complete suppression of the HPG axis have clear clinical value. Targeted partial gonadotrophin suppression, such as that afforded via kisspeptin and/or neurokinin B inhibition, has the potential to overcome the existing drawbacks of GnRH analogues although the emerging data on kisspeptin analogues (MacLean *et al.*, 2013; Scott *et al.*, 2013) demonstrate the potential for profound suppression as well.

Conclusions

The discovery of kisspeptin has transformed our understanding of the neuroendocrine signals controlling the reproductive axis. Kisspeptin coordinates GnRH secretion, mediates gonadal steroid negative and positive feedback, controls the onset of puberty, and relays information regarding the body's energy stores. The last decade has thus seen a huge resurgence in interest in neuroendocrinology, and the potential for translational application is already being explored in human studies. However, much remains to be learnt before kisspeptin can replace or be used in conjunction with GnRH and gonadotrophin analogues, the current mainstay of infertility and reproductive endocrine disorder treatments.

The mode of kisspeptin administration, as with most peptides, remains a challenge and there is thus the need for novel approaches and the development of non-peptide analogues, which is already well underway. These will also allow refinement of experimental approaches to explore physiological pathways (such as elaboration of the importance of the sex steroid environment) as well as novel treatment strategies across a wide range of conditions requiring manipulation of gonadal function. Co-administration of kisspeptin, opioid and neurokinin B modifying agents will allow fine modulation of the HPG axis that may open new therapeutic avenues.

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Authors' roles

K.S., J.T.G. and R.A.A. contributed equally to determining the scope of the review. K.S. and J.T.G. undertook the literature review. K.S. drafted the manuscript, which was edited by J.T.G. and R.A.A. All authors have approved the final manuscript for submission.

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Conflict of interest

J.T.G. serves as the International Co-ordinating Investigator for an AstraZeneca sponsored clinical trial in PCOS, as a consultant for AstraZeneca and Takeda Pharmaceuticals; and has received educational grants, speaker fees or advisory board fees from most leading pharmaceutical companies active in the field of diabetes. R.A.A. has undertaken consultancy work for AstraZeneca and Takeda Pharmaceuticals.

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ORIGINAL ARTICLE

Interactions Between Neurokinin B and Kisspeptin in Mediating Estrogen Feedback in Healthy Women

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Context: Kisspeptin and neurokinin B (NKB) are obligate for normal gonadotropin secretion, but their hierarchy is unexplored in normal women.

Objective: To investigate the interaction between kisspeptin and NKB on estrogen-regulated LH secretion.

Design: Women were treated with neurokinin-3 receptor (NK3R) antagonist followed by transdermal estradiol to induce LH secretion 48 hours later, with kisspeptin-10 or vehicle infusion during estrogen administration in a 2-way crossover study.

Setting: Clinical research facility.

Patients or other participants: Healthy females with regular menses.

Intervention(s): NK3R antagonist AZD4901 40 mg twice daily orally was taken from cycle day 4–6 for 6 days (n = 10, with 10 no treatment controls). Transdermal estradiol patches (200 μg/d) were applied after 5 days of NK3R antagonist treatment. At 24-hour estradiol treatment, women were randomized to 7-hour kisspeptin-10 (4 μg/kg/h) or vehicle iv infusion, with the alternate infusion in a subsequent cycle.

Main outcome measure(s): Plasma gonadotropin and estradiol secretion.

Results: After an initial suppression, LH secretion was increased 48 hours after estradiol treatment. Kisspeptin-10 increased LH secretion during the inhibitory phase, and LH remained elevated beyond the discontinuation of kisspeptin-10 infusion. NK3R antagonist decreased LH pulse frequency (0.5 $\pm$ 0.2 vs 0.7 $\pm$ 0.2 pulses/h, $P < .05$) and stimulated FSH response to kisspeptin-10 infusion (10.7 $\pm$ 11.0 vs 5.0 $\pm$ 3.6 IU/L, $P < .05$) with a nonsignificant rise in LH. The duration of LH response was blunted, with LH being lower at 48 hours (7.5 $\pm$ 4.8 vs 15.0 $\pm$ 11.4 IU/L, $P < .05$).

Conclusions: These data demonstrate that NKB signaling regulates GnRH/LH secretion in normal women, and is predominantly proximal to kisspeptin in mediating estrogenic positive and negative feedback on LH secretion. (*J Clin Endocrinol Metab* 101: 4628–4636, 2016)

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Abbreviations: ApEn, approximate entropy; NKB, neurokinin B; NK3R, neurokinin-3 receptor.

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Sex steroid feedback regulates the pulsatile release of hypothalamic GnRH, thereby controlling gonadotropin (LH and FSH) secretion and gonadal function (1). During the early follicular phase of the menstrual cycle, estrogen feedback is inhibitory, but during the late follicular phase, estrogenic feedback stimulates GnRH secretion, culminating in the midcycle LH surge that triggers ovulation. Neuroendocrine mechanisms involved in these pathways and the switch from negative to positive estrogen feedback in the late follicular phase remain unclear.

Kisspeptin and neurokinin B (NKB), neuropeptides partially coexpressed by a population of neurons that also express the opiate, dynorphin, are now recognized as central to the regulation of human reproduction. Patients with loss-of-function mutations in kisspeptin, NKB, or their respective receptors (kisspeptin receptor and neurokinin-3 receptor [NK3R]) show hypogonadotropic failure of pubertal progression (2–5), whereas activating mutations in kisspeptin receptor are associated with precocious puberty (6). Experimental characterization of the relative roles played by kisspeptin and NKB, as well as their functional hierarchy, has been largely carried out in nonhuman models (7–11). In patients with genetic defects inactivating NKB signaling, exogenous kisspeptin, administered using a regimen shown to be maximally stimulatory in healthy volunteers (12), restored LH pulse frequency to normal (13). This, and concordant data from animal models (7, 8), has led to the conclusion that central NKB signaling is functionally upstream of kisspeptin. Data from animal studies of administration of exogenous NKB are discordant, with both stimulatory and inhibitory effects on LH secretion being reported (9–11), whereas it elicited little effect on gonadotropin secretion in a human study (14).

In women, gonadotropin response to exogenous kisspeptin is dependent on the sex-steroid milieu (15) and is greatest in the late follicular phase of the menstrual cycle (16–18), suggesting a role for kisspeptin in the preovulatory positive estrogenic drive to GnRH/LH secretion. Exogenous kisspeptin can increase LH secretion sufficiently to induce oocyte maturation after ovarian stimulation (19), but the role of kisspeptin in physiological positive estrogen feedback is unclear. Involvement of kisspeptin in the ovulatory LH surge in rats and sheep is demonstrated by loss of the LH surge during kisspeptin receptor antagonist treatment (20, 21). The effect of kisspeptin appears largely through an increased frequency of pulsatile GnRH secretion (12, 22, 23), which preferentially stimulates LH over FSH secretion from gonadotrophs (24). Recent data from animal models indicate that administration of an NKB receptor antagonist can slow LH pulsatility (25), and this has also been demonstrated in women with polycystic ovary syndrome (26) where LH pulse frequency is often increased.

We investigated the role of kisspeptin and NKB signaling in the regulation of positive estrogen feedback in women by administration of an NKB receptor antagonist and an infusion of kisspeptin-10 during exogenous estrogen administration. We hypothesized that in this model of estrogen-induced LH secretion, kisspeptin would augment LH secretion and that pharmacological blockade of NKB signaling would reveal the functional hierarchy between kisspeptin and NKB in generating the preovulatory LH surge and in modulating GnRH/LH pulsatility.

Materials and Methods

Participants

Twenty healthy women, aged 18–45 years with regular menstrual cycles (25–35 d), were recruited from the community to this study, which was approved by South East Scotland Research Ethics Committee (reference 09/S1101/67); all volunteers provided informed written consent. Subjects were not taking steroidal contraception, had normal physical examination, and full blood count, renal function, electrolytes, liver function, and electrocardiogram were within normal limits.

Study drugs

Kisspeptin-10 was custom synthesized under Good Manufacturing Practice standards (Bachem GmbH) (12). One milligramof kisspeptin-10 was dissolved in 5-mL sterile normal (0.9%) saline immediately before infusion. The syringe and line for infusion were first coated for 30 minutes with kisspeptin-10 to minimize peptide loss from adherence to the plastic. Sterile normal saline was infused as vehicle. The specific NK3R inhibitor AZD4901, formulated as 20-mg tablets, was gifted by AstraZeneca UK. Transdermal patches releasing 200-μg 17β-estradiol per 24 hours (Janssen) were used as exogenous estradiol treatment (27).

Protocol

To standardize estrogen exposure and the onset of increased LH secretion, we used a model of follicular phase administration of transdermal estradiol (200 μg/d), which initially suppresses then at 48-hour increases LH secretion (28). In preliminary studies (Supplemental Figure 1), we confirmed that LH secretion at 48 hours is increased to the same extent if the patches were removed at 32 hours or continued till 72 hours: for the main study, therefore, patches were removed at 32 hours. Sample size was based on previous proof of concept mechanistic studies (12, 15). Twenty women were randomly allocated to NK3R antagonist (AZD4901) 40 mg oral twice daily starting from cycle day 4–6 for 6 days, or no treatment (Figure 1). Two transdermal estradiol patches were administered after 5 days (time 0 h), in the late follicular phase (cycle d 9–11, according to the day of starting AZD4901). At 24 hours of estradiol treatment, volunteers attended our clinical research facility for 8 hours. After an hour of baseline sampling, volunteers were randomized (using sealed envelopes) to receive a continuous iv infusion of kisspeptin-10 (4

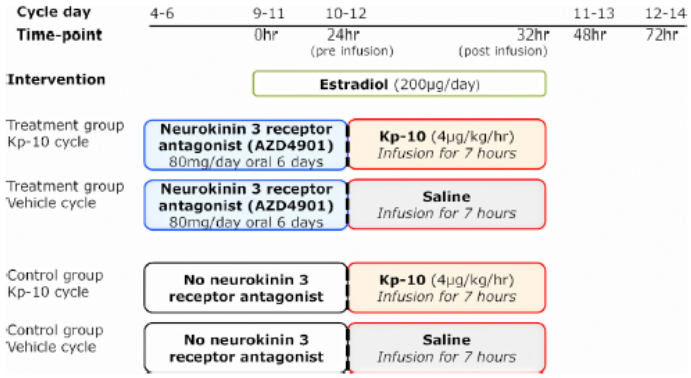

Figure 1. Study protocol diagram. Follicular phase administration of transdermal estradiol was used to induce LH secretion 48 hours later as a model of the midcycle LH surge in women. Ten healthy women were administered NK3R antagonist AZD4901 from cycle day 4–6 for 6 days, matched to 10 women having no treatment. Transdermal estradiol was applied after 5 days; 24 hours later, women were randomized to 7 hour of kisspeptin-10 (Kp-10) or vehicle infusion, returning in a subsequent cycle for the alternate infusion. Reproductive hormones were measured throughout the study and LH pulsatility assessed during 10 minutes of blood sampling for 8 hours.

µg/kg/h) or vehicle for 7 hours. In the NK3R antagonist treatment group, the last dose of AZD4901 was on the morning of kisspeptin-10 or vehicle administration. Estradiol patches were removed at the end of the infusion, ie, 32 hours after application. Volunteers attended for further measurement of reproductive hormones at 48 and 72 hours. In a subsequent menstrual cycle, all women returned to receive the alternate infusion of kisspeptin-10 or saline. Those receiving NK3R antagonist had at least 1 wash out cycle between treatment cycles. To compare the effect of exogenous vs endogenous estrogen on kisspeptin-10 response, another group of 10 women received iv kisspeptin-10 (4 µg/kg/h) infusion for 7 hours on cycle day 10–12 without exogenous estrogen treatment, with reproductive hormone measurements at equivalent time points.

Blood sampling and hormone assays

Peripheral venous blood was sampled for LH, FSH, and estradiol in the treatment group on the day of commencing NK3R antagonist and in both control and treatment groups before estradiol treatment (0 h) and then at 24, 32, 48, and 72 hours. During the 8-hour visit, blood samples were collected via an indwelling iv cannula at 10-minute intervals for assessments of LH pulsatility; FSH was measured hourly. Blood samples were centrifuged at 4°C for 10 minutes at 3000 rpm, and serum was frozen at −20°C or below until analysis. LH and FSH were determined by ELISA as previously described (12). 17β-estradiol was measured by ELISA (Demeditec Diagnostics). Inter- and intraassay coefficient of variation for all hormones was less than 5% at the concentrations measured. Lower detection limit for LH and FSH was 0.1 IU/L and for estradiol 20 pmol/L.

Statistical analysis

ANOVA was used to analyze preliminary data on LH changes with time in the model. For the primary endpoints, hormone concentrations were compared between the 4 treatment groups at specific time points using ANOVA with repeated measures as appropriate. If there was overall significance, post hoc analysis was performed with Bonferroni's correction for multiple comparisons, comparing all 4 treatments simultaneously at each time point. The relationship between the timing of peak LH and treatment was assessed by χ^2 test. Pearson correlation coefficient was computed to assess the relationship between estradiol concentrations and LH response to kisspeptin-10.

The number of LH pulses, secretory mass of LH per pulse, basal (nonpulsatile), and pulsatile (integral of dual amplitude and frequency regulation) LH secretion were identified by an established deconvolutional algorithm with cluster analysis (93% sensitivity and specificity) (29, 30) blinded to treatment allocation. Approximate entropy (ApEn), a measure of orderliness, was also estimated for the pattern of LH secretion. Deconvolutional estimates and mean hourly hormone changes were not calculated for one woman in each group, as full 8-hour sampling data were not obtained. ANOVA was used to assess changes in LH pulsatility parameters between the 4 groups, with post hoc testing as above.

Data are presented as mean ± SD. Data not normally distributed were log-transformed before statistical analysis, resulting in a distribution that approximated a normal distribution. Differences were regarded as significant at a 2-sided $P < .05$. The statistical software package GraphPad Prism (GraphPad) was used.

Results

Baseline age, Body Mass Index, and the menstrual cycle length were comparable between the subjects in the control and the treatment group, as were baseline LH, FSH, and estradiol levels in vehicle and kisspeptin-10 cycles within the group (Table 1).

Model validation for estrogen-induced LH secretion

Treatment with exogenous estrogen for 32 hours increased serum estradiol concentrations as expected ($P < .0001$) (Supplemental Figure 1). Serum LH was initially suppressed at 32 hours of estrogen treatment, then increased at 48 hours, which persisted at 72 hours (all $P < .05$ vs 0 h). FSH concentrations were significantly lower at 24 ($P < .01$) and 32 hours ($P < .0001$) but were not higher at 48 and 72 hours compared with baseline. This confirms that with this regimen, estrogenic negative feedback is followed by increased LH secretion, thus standardizing estrogen exposure and the time course of changes in LH secretion.

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Table 1. Baseline Characteristics of Women in the Control and the Treatment Group Undergoing Vehicle and Kisspeptin-10 Infusion

	Control Group			Treatment (NK3Ra) Group		*P* Value
n	10			10		
Age (y)	35 ± 5.8			35 ± 5.4		ns
BMI (kg/m^2)	25 ± 4.6			28 ± 6.9		ns
Cycle length (d)	29 ± 1.8			28 ± 1.4		ns
	Vehicle Cycle	**Kp-10 Cycle**	***P* Value**	**Vehicle Cycle**	**Kp-10 Cycle**	***P* Value**
Menstrual cycle day	9.2 ± 0.8	9.4 ± 0.8	ns	4.4 ± 0.5	4.8 ± 0.8	ns
LH (IU/L)	4.8 ± 2.1	5.6 ± 2.0	ns	5.0 ± 1.8	4.9 ± 1.8	ns
FSH (IU/L)	4.3 ± 1.2	5.3 ± 2.6	ns	6.2 ± 1.8	5.5 ± 1.3	ns
Estradiol (pmol/L)	274 ± 126	287 ± 157	ns	121 ± 54	154 ± 47	ns

Data are shown as mean + SD; BMI, Body Mass Index; Kp-10, Kisspeptin-10; ns, not significant. Note that baseline data on control and treatment groups in lower part of the table reflect sampling at different stages of the menstrual cycle.

Kisspeptin-10 stimulates gonadotropin secretion

During estrogen administration, kisspeptin-10 stimulated LH secretion to 16.4 ± 12.4 IU/L at the end of infusion vs 2.9 ± 1.0 IU/L after vehicle administration ($P <$ .0001) (Figure 2A). The time course of this response is shown in Figure 3A. Kisspeptin-10 induced LH secretion persisted beyond the discontinuation of the infusion with higher peak LH compared with controls at 48 hours (9.3 ± 1.9 vs 21.6 ± 13.0 IU/L, P = .007) (Supplemental Table 1). Clarification of the impact of exogenous estradiol on this response was demonstrated in a separate group of women receiving kisspeptin-10 infusion only in the late follicular phase without exogenous estrogen administration, who showed a similar acute increase in LH secretion correlating positively with estradiol concentration (r^2 = 0.63, P = .006), but of a shorter duration (48 h: 6.8 ± 5.8 IU/L vs 15.0 ± 11.4 with estrogen treatment, $P <$ .01) (Supplemental Figure 2). All subjects in the endogenous estrogen group had peak LH at the end of kisspeptin-10 infusion, whereas in exogenous estrogen-treated subjects, the kisspeptin-10-induced peak LH persisted beyond kisspeptin-10 infusion with 50% of women having peak LH at 32 hours and 50% at 48 hours ($P <$ .01, Supplemental Table 1).

FSH secretion was also significantly higher at the end of kisspeptin-10 infusion compared with vehicle in the control group ($P <$ .05) but not different to baseline (0 h) (Figures 2B and 3B). As expected with this model of exogenous estradiol administration, estradiol concentrations were similar in kisspeptin-10 and vehicle-infused controls (Figure 2C).

NK3R antagonist has differential effects on LH and FSH secretion

Serum LH levels did not change after 5 days of NK3R antagonist treatment (before the estradiol patches were applied) when compared with either pretreatment concentrations (pre-NK3Ra 5.0 ± 1.8 vs 5 d NK3Ra 6.6 ± 4.0 IU/L, nonsignificant [ns]) or to controls (Figure 2A). Overall, there was no difference in LH concentrations and the timing of peak LH in controls vs NK3Ra-treated women (Supplemental Table 1 and Figure 2A). To detect subtle changes in hormone secretion potentially overlooked by single time point blood sampling, analysis of hourly LH for 8 hours after dose showed that overall LH secretion was lower in NK3Ra-treated women compared with controls ($P <$ .0001) (Figure 3A), although post hoc analysis indicated no significant differences in LH levels at any individual hourly time point.

FSH concentrations appeared higher throughout treatment with NK3R antagonist compared with controls (Figure 2B) and were significantly higher in NK3Ra-treated women throughout the 8-hour period (ie, during saline infusion, $P <$ .0001) (Figure 3B). This may reflect that serum estradiol concentrations were significantly lower after 5 days of treatment with NK3R antagonist compared with controls ($P <$ .05) (Figure 2C) and comparable with estradiol levels before NK3R antagonist administration (pre-NK3Ra, 121 ± 54 vs 145 ± 87 pmol/L; after 5 d of NK3Ra, ns).

Effect of NK3R antagonist on the gonadotropin response to kisspeptin-10

NK3R antagonist nonsignificantly increased kisspeptin-10 stimulated LH secretion at 32 hours (21.6 ± 17.8 with NKB antagonist vs 16.4 ± 12.4 IU/L kisspeptin-10 alone, P = .41) (Figures 2A and 3A). The FSH response to kisspeptin-10 was, however, significantly more pronounced in the presence of NK3Ra (10.7 ± 11.0 vs 5.0 ± 3.6 IU/L at 32 h; $P <$ .05) (Figure 2B) and throughout the 7-hour infusion ($P <$ .0001) (Figure 3B).

However, NK3Ra blunted the duration of kisspeptin-10-induced LH secretion, with significantly lower LH at 48 hours (15.0 ± 11.4 vs 7.5 ± 4.8 IU/L, $P <$ .05) when compared with kisspeptin-10 infused controls, whereas FSH showed no significant difference (Figure 2). There

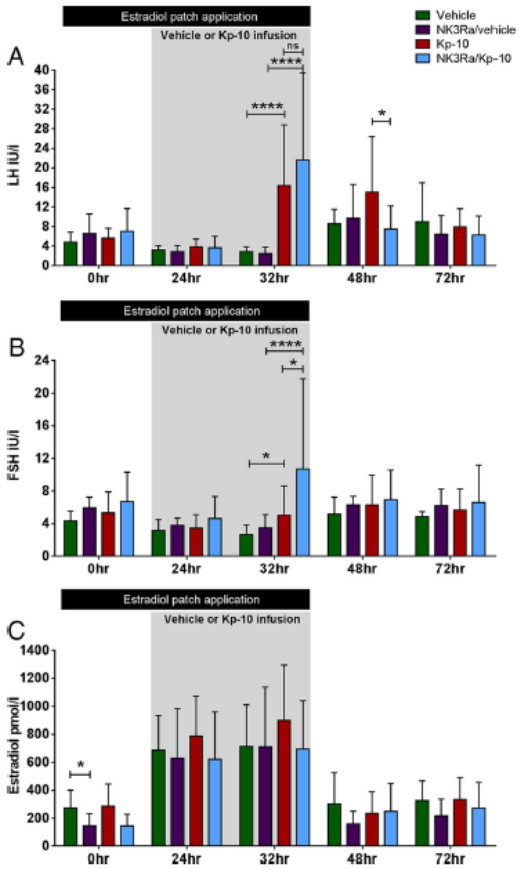

Figure 2. Comparison of mean LH (A), FSH (B), and estradiol (C) response to an infusion of kisspeptin-10 (Kp-10) and vehicle in 10 control and 10 NK3R antagonist-treated women in the model of estrogen-induced LH secretion. Two estradiol patches releasing a total of 200-μg estradiol/d were applied between 0 and 32 hours. The infusion period of kisspeptin-10 and vehicle was between 24 and 32 hours. Data presented as mean ± SD. *, $P < .05$; ****, $P < .0001$.

were related changes in the timing of the LH peak (although not statistically significant), which was at 32 hours in 9/10 NK3Ra-treated women in response to kisspeptin-10 infusion, compared with kisspeptin-10 treated controls whose LH peak timing was evenly divided between 32 and 48 hours (Supplemental Table 1).

NK3R antagonist impedes estradiol-dependent kisspeptin-10 response

The relationship between LH response to kisspeptin-10 and estradiol exposure, and the influence thereon of NK3Ra treatment, was investigated by analyzing LH concentration at the end of kisspeptin-10 infusion in relation to endogenous estradiol concentrations at 0 hours (ie, before transdermal estradiol application). There was a strong positive correlation in controls ($r^2 = 0.75$, $P = .001$) (Figure 4). However, in NK3Ra-treated women, the LH response to kisspeptin-10 showed no such relationship ($r^2 = 0.007$, ns). Very similar results were obtained when the analysis was based on estradiol concentrations after 24 hours of patch administration ($r^2 = 0.65$, $P = .005$ in controls; $r^2 = 0.03$, ns in NK3Ra-treated women).

Interaction between NK3R antagonist and kisspeptin-10 in regulating LH pulsatility

LH pulse frequency increased from 0.7 ± 0.2 pulses/h in vehicle cycle to 1.0 ± 0.2 pulses/h during kisspeptin-10 infusion ($P < .01$) (Figure 5, A and B). NK3R antagonist reduced LH pulsatility to 0.5 ± 0.2 pulses/h ($P < .05$ vs vehicle-infused controls), but administration of kisspeptin-10 to NK3Ra-treated women restored LH pulse frequency to that observed in kisspeptin-10-infused controls. Thus, although NK3Ra slowed LH pulsatility in estrogen-treated women, it did not affect the response to kisspeptin-10, indicating that kisspeptin effects are downstream of NKB signaling.

Secretory mass of LH per pulse was increased similarly during infusion of kisspeptin-10 compared with vehicle in both control ($P < .05$) and NK3R antagonist-treated women ($P < .01$) (Figure 5C). NK3Ra antagonist did not reduce LH secretory mass per pulse.

Consistent with increased LH pulse frequency, basal LH secretion decreased and pulsatile LH secretion increased during kisspeptin-10 infusion in the control group ($P < .05$ vs vehicle) (Figure 5, D and E). Basal LH secretion appeared lower in NK3Ra-treated women when compared with controls, but there was no effect on pulsatile LH secretion. Kisspeptin-10 induced the same changes in NK3Ra-treated women as in controls, with no change in basal and an increase in pulsatile LH secretion ($P < .0001$).

The regularity of LH secretory pattern was assessed by ApEn. Both kisspeptin-10 infusion and NK3Ra separately imposed greater orderliness (lower ApEn) in LH secretion ($P < .05$) (Figure 5F). This was increased further in NK3Ra-treated women during kisspeptin-10 infusion ($P < .0001$ vs NK3Ra alone) (Figure 5F).

Discussion

In a model of LH modulation by estrogen administration in women, exogenous kisspeptin-10 stimulated LH secretion, the extent of which reflected estradiol concentrations. Pharmacological blockage of NKB-NK3R signaling slowed LH pulsatility and shortened the duration of kisspeptin-mediated LH secretion with “sharpening” of the

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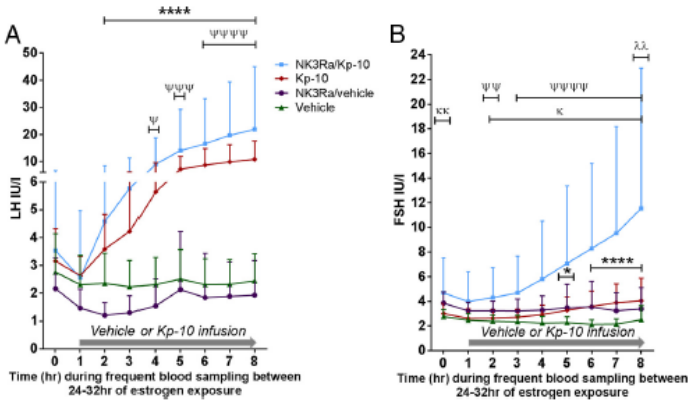

Figure 3. LH (A) and FSH (B) levels an hour before and during 7 hours of vehicle or kisspeptin-10 (Kp-10) infusion in the control group (n = 9) and in the treatment (NK3R antagonist) group (n = 9). The infusion period of kisspeptin-10 and vehicle was between 24 and 32 hours of estrogen administration. Data presented as mean ± SD. Statistical analysis by two-way ANOVA determined statistically lower LH levels between vehicle and NK3Ra-treated women ($P < .0001$), although Bonferroni's post hoc multiple comparison test found no significant changes at specific time points. For vehicle vs kisspeptin-10 infused controls: *, $P < .05$; ****, $P < .0001$. For kisspeptin-10 infusion in controls vs NK3Ra: λλ, $P < .01$. For vehicle vs NK3Ra: κ, $P < .05$; κκ, $P < .01$. For vehicle vs kisspeptin-10 in NK3Ra-treated women: Ψ, $P < .05$; ΨΨ, $P < .01$; ΨΨΨ, $P < .001$; ΨΨΨΨ, $P < .0001$.

LH response, and strikingly abolished the relationship between estradiol and LH response to kisspeptin. Taken together, these data support a central role for kisspeptin in the modulation of GnRH/LH secretion, and although NKB signaling is largely upstream of kisspeptin as previously reported (13), both pathways interact in determining the timing and characteristics of estrogenic negative and positive feedback on LH secretion.

The stimulatory effect of exogenous kisspeptin on LH secretion in women is dependent on the sex steroid environment (15–18). This response is initially limited but increases markedly in the late follicular phase of the menstrual cycle when estradiol levels are rising (16–18). For most of the cycle, GnRH and thus LH secretion are inhibited by negative feedback from estradiol (and progesterone in the luteal phase), thus the low responsiveness to kisspeptin administration in previous studies is consistent with endogenous kisspeptin signaling being suppressed by the inhibitory steroidal actions, presumably at the GnRH neuron and/or gonadotroph level. Conversely, the enhanced LH response in the later follicular phase may indicate the development of increased endogenous kisspeptin signaling in the lead up to the midcycle surge. This is supported by animal studies, where kisspeptin expression is highest after an estrogen challenge in the anteroventral periventricular nucleus (the site of positive estrogen feedback in rodents) in ovariectomized mice (31) and at the time of GnRH/LH surge in sheep (32), but is prevented by administration of kisspeptin receptor antagonist (20, 21). The present data suggests a role of estradiol in modulating LH response to kisspeptin-10 infusion, which persisted well beyond the pharmacokinetic clearance of the exogenous kisspeptin-10. The striking positive relationship between estradiol concentration in the late follicular phase and the LH response to kisspeptin-10 infusion lends further support for the involvement of kisspeptin in estrogen feedback, as recently demonstrated for kisspeptin-54 (33).

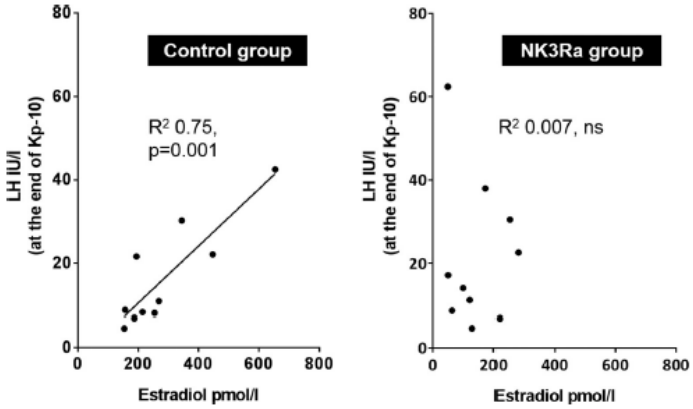

Figure 4. Correlation between endogenous estradiol and LH response to kisspeptin-10 in controls (left) and NK3R antagonist-treated (right) women. Kisspeptin-10 response on LH secretion is positively related to endogenous estradiol levels, whereas this correlation is not seen during NK3R antagonist treatment. Analysis of residuals demonstrated normal distribution.

The present data demonstrate that NK3R antagonist treatment, in an environment of high estrogenic negative feedback, reduced LH secretion and pulse frequency, whereas in the presence of kisspeptin-10 had a stimulatory effect on the secretion of both gonadotropins, but with a shorter duration of LH response. Hitherto, NK3R antagonists have been demonstrated to suppress LH secretion in states of high LH output, such as in women with Polycystic Ovary Syndrome (26), or in the ovariectomized ewe and castrate monkeys (25, 34), and in intact fe-

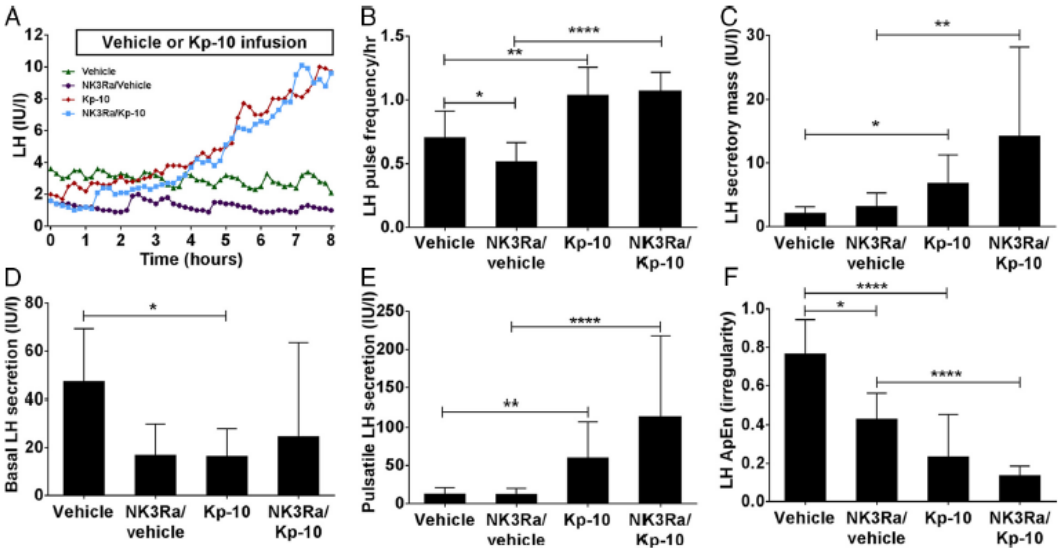

Figure 5. Analysis of 8 hour LH secretory pattern during vehicle and kisspeptin-10 (Kp-10) in controls and NK3Ra-treated women. A, Illustrative LH pulse profile from one subject undergoing vehicle (green triangles), NK3R antagonist (purple circles), kisspeptin-10 (red diamonds), and NK3R antagonist followed by kisspeptin-10 (blue squares) treatment visits. Mean LH pulse frequency (B), secretory mass of LH per pulse (C), basal (nonpulsatile) LH secretion (D), pulsatile LH secretion (E), and the relative orderliness/regularity of LH secretory pattern (F) during vehicle and kisspeptin-10 infusion with (n = 9) and without (n = 9) pretreatment with NK3R antagonist. Mean ± SD. *, $P < .05$; **, $P < .01$; ****, $P < .0001$.

male monkeys a delay of surge like, but no decrease in basal LH secretion was observed (25). Although it appears that the suppression of LH secretion by the present dose and regimen lasted only a few hours, this was sufficient to significantly lower estradiol concentrations after 5 days of NK3R antagonist treatment (ie, before the estrogenic treatment) compared with controls. Although this demonstrates a role of NKB in the regulation of LH secretion, NK3R antagonist had no effect on the timing of peak LH secretion in this model of estrogen administration, which is consistent with a lack of effect of NK3R antagonist on the estrogen induced LH surge seen in ovariectomized ewes (34). The mechanisms critical for progression to positive estrogen feedback therefore appear to be largely independent of NKB but dependent on kisspeptin, consistent with rodent neuroanatomical data (31, 35). A recent study using a different NK3R antagonist in normal women also showed a temporary suppression of LH levels lasting few hours after dosing, but no overall decrease in basal LH secretion after treatment throughout the follicular phase (34). NK3Ra did, however, delay LH surge in some women, probably as a consequence of delayed preovulatory estradiol rise, but the study did not assess the effect of NKB antagonism at the time of the switch from negative to positive estrogen feedback, when NKB might be no longer critical (36). Unlike in the present study, no effect of NK3Ra on FSH secretion was observed (35).

We have previously demonstrated that infusion of kisspeptin-10 can restore LH pulsatile secretion in men and women with inactivating mutations in NKB signaling, indicating that kisspeptin is functionally downstream of NKB in LH pulse generation (13). This is supported by the inability of the NK3R agonist senktide to stimulate LH secretion in *Kiss1r* knockout mice (7). Consistent with this overall hierarchy, NKB antagonism (active during kisspeptin-10 administration as half-life of AZD4901 is 8.5 h) (37) did not prevent the stimulatory effect of kisspeptin-10 infusion on LH secretion. NKB antagonist, however, shortened the LH response to kisspeptin-10, affecting its timing by reducing the variability of peak LH secretion, and disrupted the relationship between LH response and estradiol concentrations. These findings suggest a more complex interaction than a linear pathway between those neuropeptides at the time of the midcycle LH surge, but are also consistent with NK3R antagonist reducing endogenous kisspeptin stimulation of GnRH as a contribution to the observed effect.

LH pulse frequency increases in the late follicular phase, culminating in the midcycle LH surge (38). Exogenous kisspeptin stimulates pulsatile LH secretion (12, 13, 22, 23, 39), but its role as a potential contributor to positive estrogen feedback has not been previously investigated. In this study, the increase in LH secretion during

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kisspeptin-10 infusion included increased LH pulse frequency and mass-per-secretory pulse. This resulted in a larger proportion of total LH secretion occurring in pulsatile bursts, and the regularity of LH secretory pattern showed greater orderliness in the lead up to the stimulatory phase of response to estrogen. Deconvolution analysis also indicated changes in the nature of the pulsatile LH secretion after NK3R antagonist administration, with reduced basal secretion and ApEn, indicating a more orderly, slowed pattern of LH and by inference GnRH secretion. The increase in LH pulse frequency resulting from kisspeptin-10 infusion and the slowing in LH pulsatility with NK3R antagonist administration both increased the regularity and orderliness of LH secretion and may be the basis for the reduced variability in the timing of peak LH secretion as well as shortened duration of stimulated LH secretion in response to kisspeptin-10. Consistent with some aspects of our findings, estrogen-induced LH surges were preserved in ovariectomized NK3R antagonist-treated ewes, although the onset-to-peak time was delayed (34). In sheep, the NK3R agonist senktide increased LH secretion, resembling "surge-like" LH levels (9), whereas in monkeys, NK3R antagonist abolished LH surge, ovulation, and subsequent progesterone rise (25). Although our data primarily indicate that NKB signaling is largely upstream of kisspeptin signaling in mediating estrogenic positive effects, it clearly has a modulatory role in determining the pattern and duration of GnRH secretion during estrogen positive feedback.

A stimulatory effect of kisspeptin alone on FSH has been minimal and inconsistent in previous studies (16–18, 40) but was robustly demonstrated in this model and was not prevented by NK3R antagonist treatment. NK3R antagonist also increased FSH secretion and markedly augmented stimulation by kisspeptin-10. These findings are consistent with well-established data from animal models showing that high GnRH pulse frequency favors LH secretion, whereas low pulse frequency favors FSH secretion (24), and that this is the main drive to follicular estrogen production, the reduction in both of which (and presumed reduced inhibin production) is likely to have resulted in the observed increased FSH secretion. The differential effects of NK3Ra on FSH vs LH response to kisspeptin-10 are also similar to the effects in patients with NKB defects (13).

Although the present study has clear strengths (the use of specific NK3R antagonist, detailed LH pulse profiling and blinded pulse analysis), there are weaknesses. The sample size is small, and placebo was not administered to the control group receiving no NK3Ra. The limited LH suppression by the NK3R antagonist might be due to the small sample size and the dose of AZD4901 may be low compared with those used in animal studies, limiting the response (25). Statistical analyses included adjustment for α for multiplicity of comparisons, but studies such as these should be regarded as mechanistic explorations. Moreover, this model of LH secretion after exogenous estrogen administration may not fully replicate physiological positive estrogen feedback in the preovulatory state.

In summary, using estrogen to standardize LH secretion in women to model the midcycle LH surge, we have shown that the increase in LH secretion by kisspeptin-10 infusion is related to estradiol exposure. We show for the first time that NK3R antagonist reduced LH pulsatility in healthy women. Assessment of the interaction between kisspeptin and NKB showed that the duration of kisspeptin-mediated LH secretion was shortened by the NK3R antagonist, and the quantitative relationship with estradiol exposure abolished. These data thus indicate that NKB pathways regulate GnRH/LH secretion in women, are predominantly upstream of kisspeptin signaling in mediating estrogen feedback, but modify this kisspeptin response. This extends our understanding of these critical events in human reproduction.

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