

Investigating the Role of Kisspeptin in Human Behaviour and Physiology

Thesis submitted for the degree of Doctor of Philosophy

May 2020

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Declaration of Originality

I hereby declare that this thesis is a presentation of my original research and was written by myself. Contributions of others have been acknowledged and referenced. This work was carried out under the guidance of Dr Alexander Comninos, Dr Matt Wall and Professor Waljit Dhillon, Imperial College London.

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Abstract

The neuropeptide kisspeptin is established as a master regulator of the reproductive axis within the hypothalamus. Kisspeptin and its receptor are also extensively distributed in extra-hypothalamic brain regions and an expanding pool of animal data demonstrates that kisspeptin sits within an extensive functional and neuroanatomical framework through which it can integrate a range of internal and external cues with appropriate behavioural and neuroendocrine responses. In keeping with this, recent studies have revealed wide-reaching effects of kisspeptin on key behaviours such as olfactory-mediated partner preference, sexual responses, mood and emotions. To date, only one human study has investigated the effects of kisspeptin on extra-hypothalamic brain pathways, with findings that peripheral kisspeptin administration enhances limbic brain processing in healthy men in response to sexually arousing and couple-bonding images. In this thesis, I have used a combination of hormonal, psychometric and functional neuroimaging techniques to study the effects of kisspeptin on two currently unexplored aspects of human behaviour and their underlying brain processes; 1) olfactory and visually stimulated sexual attraction; 2) appetite and food intake. My results show that kisspeptin enhances brain processing in response to olfactory and visual cues of attraction but has no effect on brain responses to food images, with no associated changes in appetite or food intake. Collectively, the findings of this thesis generate novel human data which support and extend the animal literature regarding the role of kisspeptin in sexual attraction and related behaviours, while answering key functional and mechanistic questions concerning the role of kisspeptin in human appetite and food intake.

Declaration of Contributors

The work described in this thesis is my own. Any collaborations and assistance are stated below:

Chapter 2: I designed the study under the guidance of my supervisors; Dr Alexander Comninou, Dr Matt Wall and Professor Waljit Dhillon. I recruited participants and performed the study visits with the assistance of Dr Edouard Mills, Dr David Zargaran, Dr Mark Sykes, Dr Lysia Demetriou and Dr Julia Prague. I analysed the fMRI brain data in conjunction with Dr Lysia Demetriou and I analysed the reproductive hormones together with Dr Edouard Mills. I performed the kisspeptin radioimmunoassay with assistance from other members of the kisspeptin team.

Chapter 3: The study protocol was designed by Dr Chioma Izzi-Engbeaya and Professor Waljit Dhillon. I performed the studies and carried out hormone analyses, including radioimmunoassays together with other members of the kisspeptin team.

Chapter 4: I designed the study under the guidance of Dr Alexander Comninou, Dr Matt Wall and Professor Waljit Dhillon. I recruited participants and performed the study visits with the assistance of Dr Edouard Mills, Dr Victoria Wing, Dr Ben Coombes, Dr Tia Hunjan and Dr Layla Thurston. I analysed the fMRI brain data in conjunction with Dr Lysia Demetriou and the reproductive hormones were analysed with the assistance of Dr Edouard Mills, Dr Pei Chia Eng and Dr Layla Thurston.

Acknowledgements

This PhD has been a journey of immense personal and professional development and I am most grateful to the Medical Research Council for awarding me a Clinical Research Training Fellowship to fund this work. I have been privileged to receive outstanding supervision throughout this time and I would firstly like to thank Professor Waljit Dhillon for giving me the opportunity to conduct research in his lab and for his exceptional academic and pastoral support over the years. I am indebted to Dr Alex Comninou for his guidance and direction, which have been pivotal in shaping me into the clinical researcher that I am today. I am also extremely grateful to Dr Matt Wall, whose good humour and neuroscience expertise have been invaluable throughout my PhD.

The clinical studies carried out in this thesis required the assistance and kindness of many people. I would like to extend my thanks to staff at the Imperial College Research Facility for their support with recruitment and screening, our departmental support team for their help with grant applications, purchasing and expenses payments, and to our study participants for their time and trust. I am also extremely grateful to all my colleagues on the kisspeptin team for their help during my PhD. In particular, I have thoroughly enjoyed the good company of Dr Edouard Mills and Dr Lysia Demetriou during the early starts for study visits and long analysis periods. It has been a pleasure to be part of such a hard-working and dedicated team throughout this time.

Finally, I would like to thank my family, and especially my husband Nicholas Voo for his unwavering love and support, which continue to drive me towards achieving my goals.

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List of Abbreviations

ACC	Anterior cingulate cortex
AgRP	Agouti-related peptide
ANOVA	Analysis of variance
AVPV	Anteroventral periventricular nucleus
BAS	Behavioral activation system
BIS	Behavioral inhibition system
BMI	Body Mass Index
BNST	Bed nucleus of the stria terminalis
BOLD	Blood oxygen level dependent
CART	Cocaine- and amphetamine-regulated transcript
DREADD	Designer receptor exclusively activated by designer drugs
ER α	Oestrogen receptor alpha
FCQ	Food cravings questionnaire
fMRI	Functional magnetic resonance imaging
FSH	Follicle stimulating hormone
GABA	Gamma-aminobutyric acid
GnRH	Gonadotrophin releasing hormone
GPR54	Orphan G protein receptor 54
HPG	Hypothalamic-pituitary-gonadal axis
ICV	Intracerebroventricular
ITI	Inter-trial interval
KISS1	Kisspeptin *
KISS1R	Kisspeptin receptor *
KNDy	Kisspeptin-neurokinin B-dynorphin network
LH	Luteinising hormone
MeA	Medial amygdala
mPFC	Medial pre-frontal cortex
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
nNOS	Neuronal nitric oxide synthase
NPY	Neuropeptide Y
OFC	Orbitofrontal cortex
OHSS	Ovarian hyperstimulation syndrome
PCC	Posterior cingulate cortex
PET	Positron emission tomography
POA	Pre-optic area
POMC	Pro-opiomelanocortin
POMS2A	Profile of mood states short form for adults
ROI	Regions of interest
RP3V	Rostral periventricular area of the third ventricle
RPM	Revolutions per minute
SADI	Sexual arousal and desire inventory
SEM	Standard error of the mean

SFG	Superior frontal gyrus
SQOL	Sexual quality of life questionnaire
TE	Echo time
TOST	Two one sided t-tests
TR	Repetition time
VAS	Visual analog scale

* The standard nomenclature uses KISS1 and KISS1R in humans and Kiss1 and Kiss1r in non-human species. If both non-human and human kisspeptin are referred to in a single sentence, then KISS1 or KISS1R are used for simplicity. Italicised terms are used when referring to kisspeptin gene expression.

Chapter 1

General Introduction

1.1 The hypothalamic-pituitary-gonadal axis

Reproduction is tightly regulated by a cascade of hormonal signals originating from a highly organised series of endocrine tissues that form the hypothalamic-pituitary-gonadal (HPG) axis. Anatomically, the HPG axis comprises the hypothalamus, pituitary gland and the gonads, which are interconnected by positive and negative feedback pathways (Figure 1.1). Regulation of the HPG axis begins at the hypothalamus, which connects to sensory areas, autonomic centres, the brainstem and spinal cord (Saper & Lowell, 2014). It can be divided into anterior, middle and posterior regions, each containing several distinct nuclei:

- The anterior region contains the preoptic area (POA) and is involved in autonomic function, circadian rhythm and vasopressin release.
- The tuberal or middle region, contains several nuclei including the infundibular nucleus (the human homologue of the arcuate nucleus in rodents) which provides integrative circuitry for feeding and sexual behaviour. Its axons form a diffuse projection system (the tuberoinfundibular tract) that facilitates secretion of releasing hormones into the hypophyseal portal vessel system connecting to the anterior pituitary.
- The posterior region forms connections with the limbic system and is involved in regulating arousal and stress responses (Saper & Lowell, 2014).

Up until recently, gonadotrophin-releasing hormone (GnRH) was thought to be the central regulator of the reproductive axis. GnRH was first discovered in the 1970s as an isolate from porcine hypothalamic extracts that was capable of stimulating release of luteinising hormone (LH) and follicle-stimulating hormone (FSH) from the pituitaries of several species (Schally *et al.*, 1971; Bliss *et al.*, 2010; Brown & Roberson, 2017). GnRH cell bodies are located in the POA and infundibular nucleus (arcuate nucleus in rodents) of the hypothalamus with projections to the median eminence where GnRH is secreted into the hypophyseal portal circulation (Kaprara & Huhtaniemi, 2018). Pulsatile GnRH release was first described in ovariectomised rhesus monkeys (Carmel *et al.*, 1976). Shortly after, the same pulsatile

secretion was confirmed in humans through serial blood sampling during pituitary surgery (Antunes *et al.*, 1978). GnRH binds to the GnRH receptor on gonadotrophs in the anterior pituitary to release FSH and LH into the bloodstream, which in turn act on the ovaries and testes to stimulate sex steroid production and gametogenesis.

In addition to negative feedback from downstream gonadal steroids, GnRH secretion is also regulated by an array of central and peripheral cues such as stress, nutritional status, metabolic derangements and photoperiod (Marques *et al.*, 2000). However, when trying to elucidate the higher inputs into the regulation of GnRH, some functional limitations have been found within the GnRH neuronal network. Most notably, GnRH neurons do not express the principle oestrogen receptor (ER α) which is obligatory for sex-steroid mediated feedback on GnRH/gonadotrophin release (Yeo & Herbison, 2014). This suggests the existence of other intermediary signals that act to relay environmental, endocrine and metabolic inputs to GnRH neurons. A population of hypothalamic neurons that secrete the neuropeptide kisspeptin, have been shown to express ER α (Dubois *et al.*, 2015) and kisspeptin is now widely recognised to act upstream of GnRH as a master regulator of the HPG axis (Yeo & Herbison, 2014).

1.2 The kisspeptin family of neuropeptides

Kisspeptins are a family of neuropeptides encoded by the *KISS1* gene, mapping to chromosome 1q32. *KISS1* was first described as a cancer suppressor gene in 1996 in Hershey, Pennsylvania (Lee *et al.*, 1996), and encodes a 145 amino acid pre-polypeptide, which is then cleaved into biologically active shorter lengths. All functional kisspeptin isoforms share a common C-terminal decapeptide sequence, which renders them capable of binding and activating the kisspeptin receptor (Tena-Sempere, 2008). Kisspeptin isoforms are numbered according to the number of amino acids they contain, with four isoforms found in

human circulation: kisspeptin-54, 14, 13 and 10, with kisspeptin-54 being the principal 54-amino acid peptide product of the *KISS1* gene (Kotani *et al.*, 2001).

Early studies revolved around kisspeptin's ability to suppress the metastatic potential of melanoma and breast cancer cell lines (Lee *et al.*, 1996; Bilban *et al.*, 2004). However the abundant expression of kisspeptin in human placental extracts, and subsequent identification in the hypothalamus, pituitary and gonads suggested there was a significant role for kisspeptin in reproductive endocrinology (Ohtaki *et al.*, 2001; Bilban *et al.*, 2004; Seminara & Crowley Jr, 2008).

1.3 The kisspeptin receptor and its characteristics

The kisspeptin receptor consists of a 398-amino acid protein with seven trans-membrane domains encoded by the *KISS1R* gene located on human chromosome 19p13.3. It was initially identified as the orphan G protein receptor 54 (GPR54) when first isolated in the rat brain (Lee *et al.*, 1999). Subsequently, the human orthologue of GPR54 was cloned and named AXOR12 (Kotani *et al.*, 2001; Muir *et al.*, 2001), however it is now more commonly known as the kisspeptin 1 receptor (KISS1R). Binding of kisspeptin to its receptor causes recruitment of intracellular messengers including inositol triphosphate and diacylglycerol through activated phospholipase C leading to intracellular calcium release and activation of protein kinase C (Liu *et al.*, 2008).

The standard nomenclature uses KISS1 and KISS1R in humans and Kiss1 and Kiss1r in non-human species. If both non-human and human kisspeptin are referred to in a single sentence, then KISS1 or KISS1R are used for simplicity. Italicised terms are used when referring to kisspeptin gene expression (Gottsch *et al.*, 2009).

1.4 The role of kisspeptin in reproduction

In 2003, two seminal studies reported loss-of-function mutations within the *KISS1R* gene in individuals presenting with idiopathic hypogonadotrophic hypogonadism (de Roux *et al.*, 2003; Seminara *et al.*, 2003), confirming the crucial functional role of kisspeptin in reproduction. At the same time, generation of *Kiss1r* knockout mice demonstrated a similar phenotype of hypogonadotrophic hypogonadism with abnormalities in male and female genital development (Seminara *et al.*, 2003; Funes *et al.*, 2003). Normal levels of GnRH with preserved responsiveness to exogenous gonadotrophins were observed in these animals which indicated that kisspeptin sits upstream of GnRH at the apex of the HPG axis (Seminara *et al.*, 2003; Funes *et al.*, 2003). Later on, mutations in the *KISS1* gene were also identified in association with hypogonadotrophic hypogonadism in rodents and humans (d'Anglemont de Tassigny *et al.*, 2007; Topaloglu *et al.*, 2012). Furthermore, activating mutations of *KISS1R* have been found in individuals with precocious puberty as a result of prolonged kisspeptin receptor activation (Teles *et al.*, 2008). It is now established that kisspeptin signalling is vital for normal pubertal development. Hypothalamic expression of *Kiss1* and *Kiss1r* mRNA is upregulated at onset of puberty in rodents and primates (Han *et al.*, 2005; Shahab *et al.*, 2005). Additionally, the proportion of GnRH neurons depolarising in response to kisspeptin increases in pubertal and adult mice (Han *et al.*, 2005) suggesting that GnRH neurons become more sensitive to kisspeptin across puberty. Furthermore, exogenous kisspeptin administration in rodents and monkeys stimulates earlier onset of puberty (Navarro *et al.*, 2004; Plant *et al.*, 2006) whereas kisspeptin antagonists delay puberty in rats (Roseweir *et al.*, 2009).

1.4.1 Kisspeptin as a master regulator of the reproductive axis

The hypothalamic distribution of kisspeptin-expressing neurons varies between species. In rodents, *Kiss1* mRNA and peptide expression have been mapped to two distinct hypothalamic regions, the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus where they are in close proximity to GnRH dendrites and cell bodies (Clarkson & Herbison, 2006; Gottsch

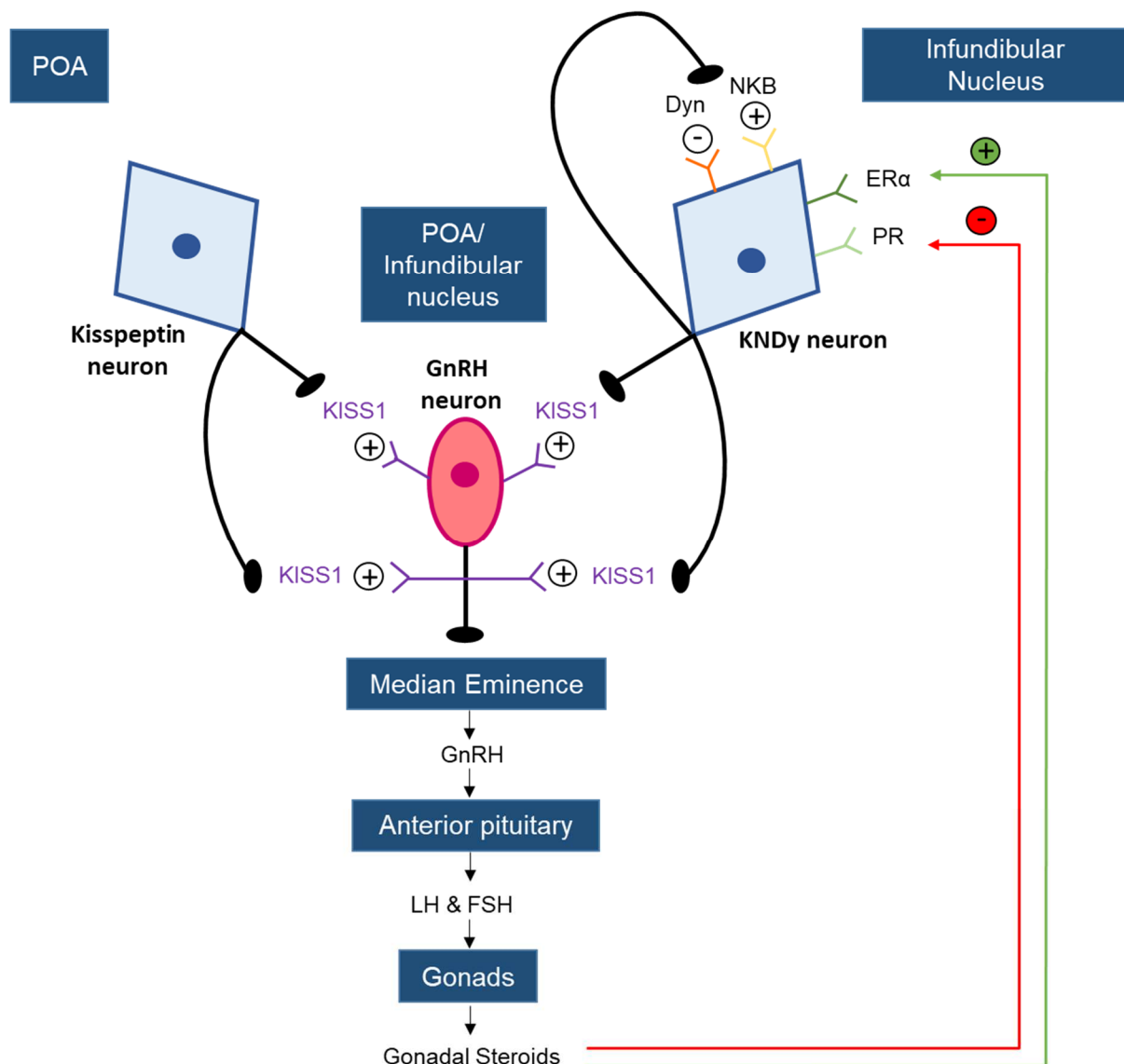
et al., 2004). In humans, KISS1-immunoreactive neurons are found in the rostral POA, and the infundibular nucleus, which is equivalent to the rodent arcuate (Hrabovszky *et al.*, 2010). Sexual dimorphism is seen across species with females expressing much larger KISS1 distribution in the AVPV and infundibular nucleus in rodents and humans respectively (Clarkson & Herbison, 2006; Hrabovszky *et al.*, 2010). Furthermore, over 90% of GnRH neurons express KISS1R (Han *et al.*, 2005) and administration of kisspeptin can induce robust GnRH/LH secretion in a wide range of species including rodents, monkeys and humans (Matsui *et al.*, 2004; Dhillon *et al.*, 2005; Messenger *et al.*, 2005; Shahab *et al.*, 2005). Conversely, administration of a GnRH antagonist to adult rodents attenuates kisspeptin's action on LH release which confirms kisspeptin as an upstream regulator of GnRH and subsequent LH secretion (Irwig *et al.*, 2004; Gottsch *et al.*, 2004).

1.4.2 The kisspeptin-neurokinin B-dynorphin (KNDy) network

Following the discovery of kisspeptin and its receptor, a subpopulation of neurons was identified within the arcuate of several mammalian species in which kisspeptin is co-localised with neurokinin B and dynorphin (Navarro *et al.*, 2009; Cheng *et al.*, 2010; Ramaswamy *et al.*, 2010). Neurokinin B is a member of the tachykinin family of peptides acting at the neurokinin-3 receptor and dynorphin is an opioid that acts at the kappa opioid receptor. Within the kisspeptin-neurokinin B-dynorphin (KNDy) network, neurokinin B and dynorphin demonstrate autoregulation of kisspeptin release, with alternate stimulation by neurokinin B and inhibition by dynorphin leading to pulsatile kisspeptin secretion (Lehman *et al.*, 2010) (Figure 1.1). KNDy neurons have been shown to project to GnRH neurons in rodents (Navarro *et al.*, 2009; Cheng *et al.*, 2010) and monkeys (Ramaswamy *et al.*, 2010). Studies of human autopsy specimens have found up to 77% of kisspeptin immunoreactive cells in the infundibular nucleus display co-expression of neurokinin B, however dynorphin immunoreactivity is sparse, which may be a result of antigen loss and sexual dimorphism (Hrabovszky *et al.*, 2010, 2012). Furthermore, tracing experiments show that KNDy neurons receive direct synaptic input from a diverse

range of upstream hypothalamic nuclei that are involved in neuroendocrine processes and sexual behaviours (Moore *et al.*, 2019). This supports a structural framework whereby KNDy neurons act as a central site for the coordination of internal and external signals that can influence the reproductive axis.

Figure 1.1: Neuroanatomy and regulation of the reproductive axis in humans



Schematic diagram showing the neuroanatomy and regulation of the reproductive axis in humans. Co-localisation of neurokinin B and dynorphin receptors on kisspeptin neurons in the infundibular nucleus act to autoregulate pulsatile kisspeptin secretion. Kisspeptin acts in turn on kisspeptin receptors located on GnRH neurons to activate the downstream cascade of GnRH and gonadotrophin release. Negative and positive feedback from gonadal steroids are relayed upstream to KNDy neurons.

POA: pre-optic area; KNDy: kisspeptin-neurokinin B-dynorphin; ERα: oestrogen receptor alpha, PR: progesterone receptor; GnRH: gonadotrophin-releasing hormone; LH: luteinising hormone; FSH: follicle-stimulating hormone; KISS1: kisspeptin. (Adapted from Skorupskaite *et al.*, 2014).

1.5 Kisspeptin administration in humans

1.5.1 Kisspeptin administration in healthy men and women

Given its established position at the apex of the HPG axis and its role as a potent secretagogue of GnRH release, a logical next step was to explore the effects of kisspeptin as a novel modulator of the human reproductive axis. In 2005, Dhillo *et al* performed the first-in-human study of kisspeptin in which a 90 minute intravenous infusion of kisspeptin-54 significantly increased LH to near maximal levels, with modest effects on FSH and testosterone release and no adverse effects (Dhillo *et al.*, 2005). Kisspeptin-10 administration in healthy males as a bolus or prolonged infusion has also been shown to increase both frequency and amplitude of LH pulses with associated testosterone rise (George *et al.*, 2011; Jayasena *et al.*, 2011). Furthermore, intravenous bolus of kisspeptin-10 is able to reset the periodicity of the LH pulse generator in men, the first known agent with the ability to manipulate the hypothalamic GnRH pulse generator in this way (Chan *et al.*, 2011). When compared to GnRH, both kisspeptin isoforms increase LH and FSH levels to a similar degree in healthy men. Although GnRH infusion leads to the greatest increase in LH and FSH, gonadotrophin levels are adequately responsive to exogenous kisspeptin and likely to be enhanced in a more physiological manner (Jayasena *et al.*, 2015).

In women, response to kisspeptin varies markedly throughout the menstrual cycle with the largest LH response in the pre-ovulatory phase when oestradiol levels are highest, followed by the luteal phase, and least in the follicular phase (Dhillo *et al.*, 2007; Chan *et al.*, 2012; George *et al.*, 2012). These findings suggest that oestradiol may enhance gonadotrophin response to exogenous kisspeptin, which is consistent with *in vitro* and rodent studies showing enhanced Kiss1 expression and augmented GnRH response to kisspeptin in the presence of oestradiol (Smith *et al.*, 2006b; Tonsfeldt *et al.*, 2011).

In summary, the above studies successfully translated animal data into humans, confirming the potency of kisspeptin in stimulating gonadotrophin release in healthy men and women. Additionally, a novel understanding of kisspeptin physiology in humans was revealed, such as the potential for kisspeptin to reset the GnRH pulse generator in men and the variable response to exogenous kisspeptin across the menstrual cycle in women. Importantly, no adverse events were reported in these studies and exogenous kisspeptin has not been associated with changes in blood pressure or other vital cardiovascular parameters in humans (Nijher *et al.*, 2010)

1.5.2 Kisspeptin administration in disorders of reproduction

Kisspeptin administration has been shown to effectively stimulate the reproductive axis in a more physiological manner when compared to GnRH receptor agonists or gonadotrophin preparations. Together with its excellent safety profile in human studies, kisspeptin represents a novel tool for the treatment of reproductive disorders. To date, stimulation or suppression of the HPG axis by kisspeptin or kisspeptin receptor agonists have been investigated as potential treatment options in a number of reproductive conditions including hypogonadism, anovulation, and infertility (Matsui & Asami, 2014).

Ovulation induction

Manipulation of LH pulsatility by kisspeptin has been well demonstrated in animal models and healthy human subjects. Persistent exposure to kisspeptin-54 in healthy women during the follicular phase causes an earlier onset of the LH peak, raising the possibility that kisspeptin may be a useful ovulation trigger for women undergoing treatment for infertility (Jayasena *et al.*, 2013). In 2014, this was investigated in a proof-of-concept study in which a single subcutaneous injection of kisspeptin-54 at four different doses was given to women undergoing *in vitro* fertilisation. Egg maturation was observed at each dose of kisspeptin-54, with a dose-dependent increase in mean number of mature eggs after kisspeptin induction (Jayasena *et al.*, 2014a). In addition to its role as an alternative ovulation inducer, kisspeptin

may have advantages over human chorionic gonadotrophin as a more physiological mediator of the LH surge. This is particularly relevant for the prevention of ovarian hyperstimulation syndrome (OHSS), a life-threatening complication of fertility treatment that can occur as a consequence of excessive stimulation of the ovaries. In a further *in vitro* fertilisation study using kisspeptin-54 in women at high risk of OHSS, Abbara *et al* showed similar rates of biochemical pregnancy (63%), clinical pregnancy (53%) and live birth rates per transfer (45%) with no incidence of moderate, severe or critical OHSS in comparison to conventional fertility treatment (Abbara *et al.*, 2015).

Hypothalamic amenorrhoea

Hypothalamic amenorrhoea is a functional condition comprised of markedly decreased GnRH pulsatility associated with a preferential decline in LH versus FSH and low ovarian follicular activity (Couzinet *et al.*, 1999). Administration of a single subcutaneous injection of kisspeptin-54 to women with hypothalamic amenorrhoea led to a four-fold higher response in LH compared to healthy women in the follicular phase of the menstrual cycle (Jayasena *et al.*, 2009). Furthermore, intravenous infusion of kisspeptin-54 increased both basal and pulsatile LH secretion in women with hypothalamic amenorrhoea (Jayasena *et al.*, 2014b). However, when twice daily kisspeptin-54 was administered in a chronic manner, tachyphylaxis was observed, whereby sustained kisspeptin exposure was associated with desensitisation in gonadotrophin response. In contrast, healthy female subjects given the same dosing protocol during their follicular phase displayed minimal down-regulation of gonadotrophins (Jayasena *et al.*, 2013). Subsequently a GnRH bolus was given, and women who no longer responded to kisspeptin-54 maintained responsiveness to GnRH. This interesting observation implies that the level of desensitisation occurs at the hypothalamus rather than at the pituitary gonadotroph (Jayasena *et al.*, 2009).

Hyperprolactinaemia

Hyperprolactinaemia is a common cause of hypogonadotrophic hypogonadism in men and women (Molitch, 2010). Excess prolactin suppresses the HPG axis at the hypothalamus as evidenced by reversal of hypogonadism and infertility in hyperprolactinaemic patients treated with pulsatile GnRH (Bouchard *et al.*, 1985; Lecomte *et al.*, 1997). However prolactin receptors are absent from the majority of GnRH neurons (Grattan *et al.*, 2007) but are present on arcuate kisspeptin neurons (Kokay *et al.*, 2011; Li *et al.*, 2011), which suggests that prolactin acts through kisspeptin to exert its effect on the HPG axis. Consistent with this, raised prolactin levels in female rodents are associated with reduced arcuate Kiss1 mRNA expression (Sonigo *et al.*, 2012) and lower kisspeptin levels (Araujo-Lopes *et al.*, 2014). Moreover, administration of kisspeptin to prolactin-treated mice leads to restoration of gonadotrophin secretion and ovarian cyclicity (Sonigo *et al.*, 2012). In a small human study, administration of kisspeptin-10 to two female subjects with cabergoline-resistant microprolactinomas significantly increased LH and FSH levels with increased LH pulses (Millar *et al.*, 2017). Taken together, these early data suggest that kisspeptin may be a therapeutic option to restore fertility in hyperprolactinaemia.

Hypogonadotrophic hypogonadism secondary to Type 2 Diabetes Mellitus

Up to half of men with type 2 diabetes mellitus exhibit subnormal testosterone levels with inappropriately low gonadotrophin concentrations (Dandona & Dhindsa, 2011). Hypothalamic Kiss1 expression is suppressed in rat models of diabetes, and exogenous kisspeptin administration can correct gonadotrophin levels in these animals (Castellano *et al.*, 2006, 2009) suggesting that the neuroendocrine mechanisms contributing to diabetes-related hypogonadism occur at or above the level of kisspeptin neurons. This was explored in a small human study involving five diabetic hypogonadal men with mean body mass index (BMI) 40 kg/m². An intravenous infusion of kisspeptin-10 given over 11 hours stimulated serum LH, increased LH pulse frequency and testosterone secretion (George *et al.*, 2011). This early data suggests that kisspeptin-based therapies could have potential in treating hypogonadism

secondary to metabolic derangements such as diabetes and could potentially restore testosterone levels to a more physiological range compared with exogenous testosterone therapy (Swerdloff *et al.*, 2000).

In summary, kisspeptin isoforms modulate the HPG axis in a potent and safe manner in humans. Clinical trials and translational studies are ongoing to explore how kisspeptin-based therapies can be used to treat an array of reproductive disorders. However, kisspeptin and its receptor are also localised in several areas of the brain not directly involved in reproduction, and exploration of its roles in these areas may unveil important non-reproductive functions of kisspeptin.

1.6 Extra-hypothalamic distribution of kisspeptin and its receptor in the brain

Since the discovery of its central role in reproduction, numerous studies have been conducted to map kisspeptin distribution in the brain. The vast majority of studies have focussed on localising kisspeptin expression in the hypothalamus where kisspeptin fibres are now known to be concentrated in key reproductive regions in rodents, ruminants, primates and humans (Lehman *et al.*, 2013). To date, only a handful of studies have explored extra-hypothalamic expression of kisspeptin and its receptor, however available data from rats, mice and humans demonstrate an extensive network of KISS1 and KISS1R fibres throughout the brain, whose functions are not yet fully understood (Findings below are summarised in Table 1.1).

1.6.1 *Kiss1 and Kiss1r brain expression in rodents*

Kiss1 expression

Using in-situ hybridisation, Kiss1 mRNA has been identified in the medial amygdala (MeA) and bed nucleus of the stria terminalis (BNST) of adult male mice (Gottsch *et al.*, 2004).

Following on from this, a study in female mice using polyclonal rabbit antibody directed towards murine kisspeptin found Kiss1 immunoreactivity in the MeA and BNST as well as the paraventricular thalamus, locus coeruleus and the periaqueductal grey (Clarkson *et al.*, 2009). Kiss1 neurons have also been identified in the MeA and BNST in rats (Kim *et al.*, 2011; Xu *et al.*, 2012). Interestingly, MeA Kiss1 populations display sexual dimorphism with greater MeA Kiss1 expression in males compared to females in dioestrus. In females, MeA Kiss1 expression varies throughout the oestrous cycle with highest levels at proestrus, indicating that MeA Kiss1 expression is sex steroid dependent (Kim *et al.*, 2011). More recently, Pineda and colleagues used retro- and anterograde tracers and viral transfection methods in male mice to map kisspeptin fibre projections from the MeA to GnRH-releasing neurons in the POA and also to the mitral cells of the accessory olfactory bulb, whose function is to relay pheromonal cues (Pineda *et al.*, 2017). Within the MeA, kisspeptin neurons are also found in close apposition with dopaminergic and vasopressinergic neurons, which are implicated in social bonding and attachment behaviour (Pineda *et al.*, 2017). Localisation of Kiss1 fibres within these areas provides a neuroanatomical framework in which kisspeptin may act as an intrinsic link to integrate social and olfactory cues with the HPG axis.

Kiss1r expression

The kisspeptin receptor also shows extensive distribution throughout the rodent brain. This was first reported in rats where northern blot and in-situ hybridisation were used to identify Kiss1r mRNA in numerous extra-hypothalamic brain regions including the pons, midbrain, thalamus, hippocampus, amygdala, frontal cortex and striatum (Lee *et al.*, 1999). In a Kiss1r LacZ knock-in mouse model whereby Kiss1r is visualised by histochemical Xgal staining for β -galactosidase (the product of LacZ), the highest density of Kiss1r expression was found in the dentate gyrus of the hippocampus (Herbison *et al.*, 2010). This technique also demonstrated Kiss1r expression in the septum, anteroventral nucleus of the thalamus, periaqueductal grey, pontine nuclei and dorsal cochlear nuclei (Herbison *et al.*, 2010).

1.6.2 *KISS1 and KISS1R brain expression in humans*

KISS1 expression

Most human studies so far have concentrated on mapping kisspeptin expression in the hypothalamus (Hrabovszky *et al.*, 2010; Taziaux *et al.*, 2016). To date, the seminal work by Muir *et al* remains the only study to describe KISS1 distribution across a range of human brain tissues (Muir *et al.*, 2001). Using quantitative reverse transcriptase – polymerase chain reaction they identified KISS1 mRNA in several key limbic regions including the amygdala, caudate, hippocampus, parahippocampal gyrus, globus pallidus, nucleus accumbens, putamen, striatum and thalamus in the brains of two men and two women (Muir *et al.*, 2001).

KISS1R expression

As with KISS1 expression, human studies of KISS1R are also extremely limited. In 2001, both Muir *et al* and Kotani *et al*, working in two independent groups published findings of KISS1R expression across a range of human brain regions. Their results were in close agreement and showed that human KISS1R distribution overlapped with KISS1 expression in many limbic regions including the amygdala, caudate, hippocampus, parahippocampal gyrus, globus pallidus, nucleus accumbens, putamen, striatum and thalamus (Muir *et al.*, 2001; Kotani *et al.*, 2001).

In summary, kisspeptin and its receptor show extensive brain distribution outside of the hypothalamus in rodents and humans. This presents the possibility that kisspeptin has important roles in limbic brain networks and that kisspeptin-signalling in these areas may integrate limbic processes (e.g. sexual, emotional and reward) with behavioural outputs and the reproductive axis.

Table 1.1 Distribution of extra-hypothalamic KISS1 and KISS1R expression

Species	Rat		Mouse		Human	
References	(A)	(B)	(C - F)	(G)	(H)	(H – I)
	Kiss1	Kiss1r	Kiss1	Kiss1r	KISS1	KISS1R
Accessory Olfactory Bulb			✓			
Amygdala	✓	✓	✓		✓	✓
Bed Nucleus of the Stria Terminalis	✓		✓			
Caudate nucleus					✓	✓
Cerebellum				✓	✓	✓
Cingulate gyrus					✓	✓
Dorsal Cochlear Nucleus				✓		
Frontal Cortex		✓			✓	✓
Globus Pallidus					✓	✓
Hippocampus		✓		✓	✓	✓
Locus Coeruleus		✓	✓		✓	✓
Medulla		✓	✓	✓	✓	✓
Nucleus Accumbens					✓	✓
Periaqueductal Grey		✓	✓	✓		
Pons		✓		✓		
Primary Olfactory Cortex		✓				
Putamen					✓	✓
Striatum					✓	✓
Substantia Nigra					✓	✓
Thalamus		✓	✓	✓	✓	✓
Ventral Tegmental Area		✓				

Summary Table of KISS1 and KISS1R brain distribution outside the hypothalamus

A: (Xu *et al.*, 2012) B: (Lee *et al.*, 1999) C: (Gottsch *et al.*, 2004) D: (Clarkson *et al.*, 2009) E: (Kim *et al.*, 2011) F: (Pineda *et al.*, 2017) G: (Herbison *et al.*, 2010) H: (Muir *et al.*, 2001) I: (Kotani *et al.*, 2001)

1.7 Overview of the limbic system

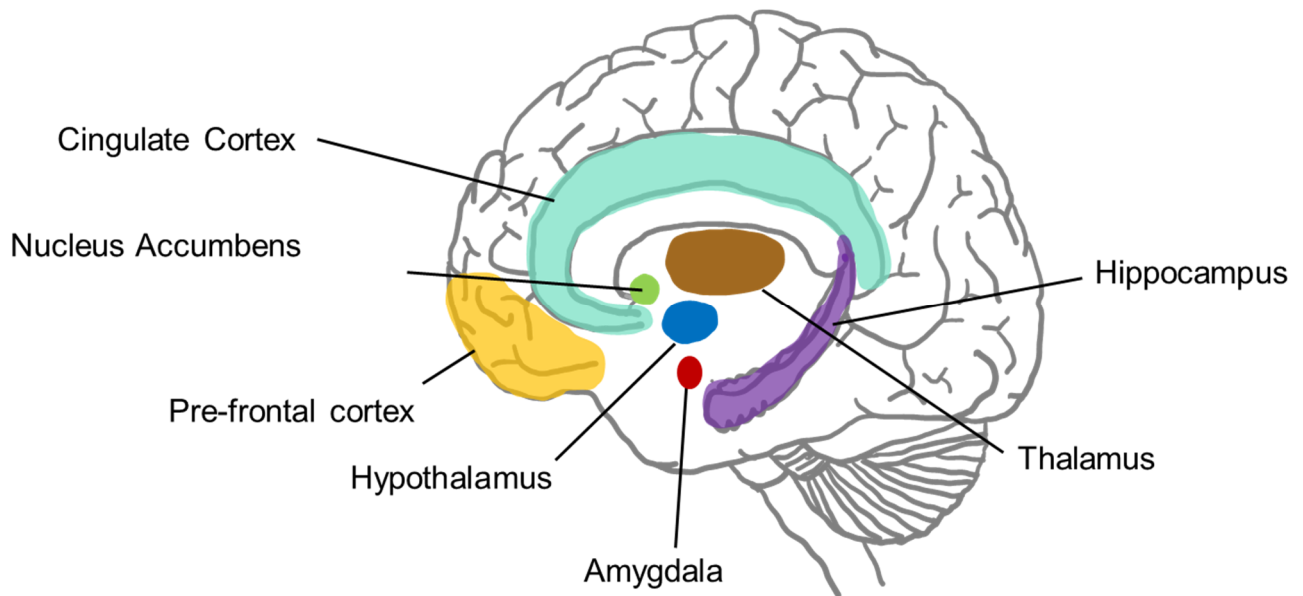
First described by Thomas Willis in 1664, the limbic system is a highly conserved functional brain network common to all mammals and is often thought of as the “emotional brain”. The currently accepted composition of the limbic system is derived from Paul MacLean’s proposal in 1952 (MacLean, 1952), and consists of several functionally interconnected cortical and subcortical structures that act as a command centre for processing sensory stimuli to allow the experience and expression of emotions beyond stereotyped reactions (Phelps & LeDoux, 2005). Maclean’s model includes the orbitofrontal cortex, hippocampus, cingulate cortex, amygdala, hypothalamus, thalamus and the ventral striatum (i.e. nucleus accumbens) (Figure 1.2). Within the limbic brain network, several structures are thought to be particularly instrumental in translating sensory stimuli into emotional and behavioural outputs. These include the amygdala, pre-frontal cortex, hypothalamus, and the cingulate cortex (Adolphs *et al.*, 1994; Baird *et al.*, 2004).

The amygdala is a small almond shaped structure lying deep to the antero-inferior temporal lobe. Animal studies have established the amygdala as a central hub for processing social signals of emotion (Wood, R I, Coolen, 1997; Rolls, 2015). In primates, a subset of amygdala neurons respond primarily to different faces (Leonard *et al.*, 1985), furthermore amygdala lesions in humans lead to impairments in response to facial expressions and other social signals (Adolphs *et al.*, 1994). The amygdala is also implicated in sexual brain processing, with positive association between sexual drive and amygdala volume in humans (Baird *et al.*, 2004). Additionally, functional magnetic resonance imaging (fMRI) demonstrates enhanced amygdala activity in response to viewing sexually arousing images in men and women (Hamann *et al.*, 2004). Within the amygdala, different sub-populations have been implicated in the processing of multi-modal sensory inputs. For example, the medial amygdala has been shown to respond to opposite-sex olfactory signals, and the posterior amygdala has a key role

in processing food cues and integrating these inputs into behavioural and endocrine responses (King *et al.*, 1994; Buchanan *et al.*, 2003; Keshavarzi *et al.*, 2014).

Other important areas include the pre-frontal cortex, which is involved in decision-making and works in concert with the amygdala to learn the emotional and motivational value of stimuli (Rolls, 2015). Current thinking marks the cingulate cortex as a conduit for visceral, attentional and emotional information, channelling signals between areas such as the amygdala and pre-frontal cortex to modulate limbic responses to emotional stimuli (Dalglish, 2004). Finally, the hypothalamus coordinates many multi-functional homeostatic mechanisms, including emotional and behavioural responses. Behavioural functions of the hypothalamus have been demonstrated in rats whereby hypothalamic stimulation leads to increased motivation towards reward (Olds & Milner, 1954). This and later work suggest that the hypothalamus is part of an extensive reward network in the brain involving the amygdala, the pre-frontal cortex and the ventral striatum. Numerous electrical stimulation studies have identified further roles for the hypothalamus in motivational responses towards sex and hunger (Dalglish, 2004). The amygdala and hypothalamus are also highly interlinked and electrical stimulation of the amygdala in rats and cats leads to increased gonadotrophin secretion (Layton *et al.*, 1981; Sirett *et al.*, 1986). This demonstrates that the amygdala-hypothalamus connection serves as a conduit between emotion-processing brain centres and reproductive neuroendocrine pathways.

Figure 1.2: Structures within the human limbic system



Schematic representation of the key structures outlined in Maclean's model of the human limbic system.

1.8 Functional links between limbic kisspeptin activity and the reproductive axis

Cre-dependent labelling has identified anatomical connections between kisspeptin neurons in the amygdala and GnRH neurons in the POA (Pineda *et al.*, 2017), and direct infusion of kisspeptin-10 into the MeA of male and female rats increases plasma LH levels in a dose-dependent manner (Gresham *et al.*, 2016; Comninou *et al.*, 2016). Furthermore, direct administration of a kisspeptin receptor antagonist into the MeA reduces plasma LH and LH pulse frequency in female rats (Comninou *et al.*, 2016). In addition, kisspeptin receptor antagonism in the MeA delays puberty onset in female rats and disrupts oestrous cyclicity with reduced incidence of LH surges in adults (Adekunbi *et al.*, 2017). In male mice, chemogenetic activation of MeA kisspeptin neurons using Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) leads to a two-fold rise in LH levels (Aggarwal *et al.*, 2018). These data demonstrate that extra-hypothalamic kisspeptin signalling can directly modulate the reproductive axis.

1.9 Kisspeptin and sexual behaviour

1.9.1 Male sexual behaviour

The MeA is a key brain region in sexual behaviour and lesions in the MeA suppress male copulatory behaviours such as erections and intromissions in rats (Kondo & Arai, 1995; Kondo *et al.*, 1997). Although androgen receptors are present in the MeA (Cooke *et al.*, 2003), direct administration of androgens into the MeA does not lead to spontaneous erections (Bialy & Sachs, 2002) suggesting that additional factors are required to stimulate this physiological pathway. Gresham *et al.* hypothesised that kisspeptin may play a role in MeA control of male sexual behaviour. They administered microinjections of kisspeptin directly into the MeA of male rats and observed dose-dependent stimulation of ex-copula erections, an effect which

was blocked by pre-treatment with a kisspeptin receptor antagonist (Gresham *et al.*, 2016). Furthermore, when kisspeptin was infused into the intracerebroventricular (ICV) space, no erections were observed (despite comparable rises in circulating LH) which demonstrates site-specificity of the MeA for kisspeptin's effects on rodent erections and suggests its role in this aspect of sexual behaviour is independent of downstream GnRH, LH or testosterone (Gresham *et al.*, 2016).

In the first study to investigate the effects of kisspeptin on human sexual function, Comninou *et al.* used fMRI to map limbic brain processing in response to sexually stimulating images. In a randomised, double-blinded, two-way crossover, placebo-controlled study, 29 healthy heterosexual men received an intravenous infusion of kisspeptin or vehicle (placebo) while viewing sexual, non-sexual, neutral and negative still images during a fMRI scan. The results showed that kisspeptin administration enhanced brain activity in several key limbic and paralimbic regions involved in sexual and emotional processing that are also known to express kisspeptin receptors. Additionally, kisspeptin's enhancement of limbic brain activity was associated with reward measures, improved positive mood, and reduced sexual aversion, providing key functional relevance (Comninou *et al.*, 2017). Furthermore, kisspeptin has also been found to modulate resting brain connectivity to enhance sexual and emotional processing (Comninou *et al.*, 2018).

1.9.2 Female sexual behaviour

In rodents, the lordosis reflex is a sign of female sexual receptivity and is regarded as a pre-copulatory behaviour. In *Kiss1* knockout female mice, there is significant impairment of lordosis behaviour, which is rescued by a single peripheral injection of kisspeptin (Hellier *et al.*, 2018). Peripheral and direct ICV injection of kisspeptin can also induce the lordosis reflex in wildtype adult females (Hellier *et al.*, 2018). A population of kisspeptin neurons in the rostral periventricular area of the third ventricle (RP3V) appear to have a central role in this behaviour as selective ablation of RP3V neurons leads to profound deficits in lordosis, whereas

optogenetic stimulation of these neurons activates lordosis behaviour (Hellier *et al.*, 2018). Moreover, kisspeptin's effects on lordosis appear to be GnRH-independent as GnRH is not able to induce lordosis in *Kiss1* knockout females (Hellier *et al.*, 2018). Given the evidence that GnRH is not requisite for certain kisspeptin-directed sexual behaviours (Gresham *et al.*, 2016; Hellier *et al.*, 2018), there is a need to explore other potential candidates that may mediate the downstream effects of kisspeptin in the limbic system. Indeed, tract-tracing demonstrates that RP3V kisspeptin neurons project to another population of kisspeptin cells in the ventrolateral part of the ventromedial hypothalamus, which express neuronal nitric oxide synthase (nNOS) (Hellier *et al.*, 2018). To assess the functional significance of this, peripheral kisspeptin was administered to nNOS knockout females where it failed to stimulate lordosis. However, co-administration of kisspeptin with a nitric oxide donor was able to restore lordosis behaviour (Hellier *et al.*, 2018), which suggests nitric oxide has an essential downstream role in the kisspeptin-lordosis pathway.

To date, there have been no human studies of kisspeptin's effects on female sexual behaviour.

1.10 The role of kisspeptin in olfaction

Olfaction underpins many important mating behaviours in mammals through the detection of chemosignals, also known as pheromones. These are volatile substances secreted externally by an individual and received by a second individual of the same species leading to specific changes in behaviour and endocrine status (Petrulis, 2013). The accessory olfactory bulb is the primary centre for detecting and processing information from chemosignals in animals (Parkes & Bruce, 1961). Using immunofluorescence histochemistry in adult male rats, *Kiss1* neurons have been visualised in the mitral cell layer of the accessory olfactory bulb (Pineda *et al.*, 2017). Furthermore, these *Kiss1* fibres connect bi-directionally with *Kiss1* neuronal fibres in the MeA which in turn, make connections with hypothalamic GnRH neurons in the

POA (Pineda *et al.*, 2017). The existence of this neuro-anatomical framework suggests that kisspeptin is a potential integrator of olfactory pheromonal cues with the reproductive axis.

1.10.1 Kisspeptin neurons respond to opposite-sex olfactory stimuli

In seasonal breeders such as goats and sheep, the introduction of a stimulus male can override the normal suppressive effects of oestradiol in anoestrous females. Termed the “ram effect”, this sociosexual stimulus is now known to be mediated by male pheromones detected by the female (Fabre-Nys *et al.*, 2015). Using electrophysiology recording electrodes in ovariectomised goats exposed to a sexually mature male, Murata *et al.* demonstrated significant rise in arcuate kisspeptin neuronal activity with concurrent LH pulse generation (Murata *et al.*, 2011). Similarly, when anoestrous ewes are exposed to a novel male, increased activity in arcuate kisspeptin neurons occurs with associated increases in LH amplitude and pulse frequency (De Bond *et al.*, 2013). In female rodents (non-seasonal breeders), increased kisspeptin activity is observed in the AVPV in response to male odours with associated augmentation of the LH surge (Watanabe *et al.*, 2017) and increased lordosis behaviour (Hellier *et al.*, 2018).

Sexual dimorphism is present, with male mice exposed to female odours showing no changes in AVPV or arcuate kisspeptin activity. Instead, a two-fold rise in the number of active MeA Kiss1 neurons is observed with concomitant rise in LH (Aggarwal *et al.*, 2018). Together with neuroanatomical evidence connecting amygdala Kiss1 neurons to the accessory olfactory bulb and POA (Pineda *et al.*, 2017), this demonstrates that MeA kisspeptin activity is central to the integration of sexually-relevant olfactory stimuli with limbic pathways and sexual behaviours in male rodents.

1.10.2 Kisspeptin and olfactory-mediated partner preference

Kisspeptin and its cognate receptor are integral to olfactory-mediated partner preference. *Kiss1r* knockout male mice display loss of olfactory preference for an oestrous female despite

normosmia, which is not rescued by testosterone replacement (Kauffman *et al.*, 2007). Likewise, ovariectomised, hormone-primed *Kiss1* null female mice lack male-directed olfactory preference, which is restored following a peripheral injection of kisspeptin (Hellier *et al.*, 2018). Consistent with the sexual dimorphism seen within *Kiss1* neuronal populations responding to opposite-sex odours (Watanabe *et al.*, 2017; Hellier *et al.*, 2018; Aggarwal *et al.*, 2018), activation of MeA kisspeptin neurons doubles the time male mice spend investigating an oestrous female, whereas ablation of AVPV kisspeptin neurons in female mice results in loss of male-directed olfactory preference (Adekunbi *et al.*, 2018; Hellier *et al.*, 2018).

Taken together, this demonstrates that amygdala and hypothalamic kisspeptin populations are essential components in the neural circuitry integrating olfactory cues with appropriate behavioural outputs such as sexual partner preference.

Olfactory performance is also significantly correlated with human sexual function (Ottaviano *et al.*, 2013; Özmen *et al.*, 2016) with reported improvements in sexual function following treatment of chronic nasal disorders (Gunhan *et al.*, 2011). However, kisspeptin's role as an integrator of olfaction, behaviour and reproduction has not yet been examined in humans.

1.11 Kisspeptin's role in audition

Vocal signals are another key sensory modality in sexual communication and mate attraction (Charlton *et al.*, 2018). Male rodents emit song-like ultrasonic vocalisations to convey information regarding sexual motivation and to attract a sexually receptive female (Dombret *et al.*, 2017). Auditory signals are integrated with other sensory cues such as olfactory signals to regulate mating behaviour and reproductive function (Asaba *et al.*, 2014). A study exploring reproductive outcomes associated with male ultrasonic vocalisations in mice found significantly increased arcuate *Kiss1* neuronal activity in females following exposure to male ultrasonic vocalisations compared with noise control (Asaba *et al.*, 2017). This indicates

another potential role for kisspeptin-signalling in the integration of auditory cues with the reproductive axis.

1.12 Kisspeptin's role in mood and emotions

1.12.1 Depression

Animal models have demonstrated a role for kisspeptin in the modulation of depression and fear. Tanaka *et al* used a modified forced swim test to assess responsiveness to stress as a marker of depressive state, whereby immobility in water is considered an indicator of depressive-like behaviour which can be ameliorated by pre-treatment with known anti-depressant therapies (Tanaka *et al.*, 2013). Mice received either vehicle or a single ICV dose of kisspeptin prior to the forced swim test. The results showed that kisspeptin significantly reduced immobility time in a dose dependent manner. Additionally, the investigators pre-treated mice with serotonergic, acetylcholine, adrenergic, dopaminergic and gamma amino-butyric acid (GABA) blockers and found that alpha2 adrenergic and serotonin receptor blockade partially reversed kisspeptin's effects on immobility time, indicating that kisspeptin may act through adrenergic and serotonin pathways to mediate its mood effects (Tanaka *et al.*, 2013).

In humans, peripheral administration of kisspeptin reduces negative mood in healthy men. Furthermore on viewing non-sexual bonding images kisspeptin enhances amygdala neuronal activity, which correlates with improvements in positive mood (Comninou *et al.*, 2017).

1.12.2 Anxiety

In addition to its antidepressant effects, studies reveal that kisspeptin-signalling modulates anxiety-like behaviours. In mice, selective stimulation of MeA kisspeptin neurons using the pharmacosynthetic DREADDs approach, significantly enhances the duration of social

interaction and exploratory time in the open arm of an elevated plus maze test suggesting reduced anxiety (Adekunbi *et al.*, 2018). However, other studies have observed anxiogenic effects of kisspeptin. In male rats, central administration of kisspeptin evokes a preference for the closed arms of an elevated plus maze test, with corresponding upregulation of spontaneous locomotor activity, and increased basal corticosterone levels indicating anxiety-like behaviour (Csabafi *et al.*, 2013). Conversely, global *Kiss1r* knockout mice with selective preservation of *Kiss1r* in GnRH neurons (thus ensuring normal testosterone levels) spend twice as much time in the open arms of an elevated plus maze indicating less anxiety (Delmas *et al.*, 2018), thus these data indicate that kisspeptin-signalling may potentiate anxiogenic neural circuits.

Meanwhile, other studies conclude that kisspeptin has no effect on anxiety. In male rats, intraperitoneal administration of kisspeptin does not alter basal or stress-induced hypothalamic-pituitary-adrenal axis activity as measured by plasma corticosterone and adrenocorticotrophic hormone levels (Rao *et al.*, 2011). Central administration of kisspeptin to male rats has no effect on stress-related behaviours (Thompson *et al.*, 2004) and consistent with this, peripheral administration of kisspeptin to healthy men does not affect psychometric measures of anxiety or circulating cortisol levels (Comninou *et al.*, 2017).

Thus, the role of kisspeptin in anxiety is conflicted and may reflect differences in routes of administration, experimental paradigm or species employed. In view of this, further studies are necessary to clarify the role of kisspeptin in anxiety-like behaviour.

1.12.3 Fear

The role of kisspeptin in fear responses has been predominantly examined in fish. In a study employing an alarm-substance-evoked fear response in Zebrafish, intracranial administration of kisspeptin dose-dependently diminished behavioural parameters of fear (such as erratic movements and freezing behaviour) with associated reduction in serotonin-related gene

expression (Ogawa *et al.*, 2014). Further mechanistic studies have identified 5-HT1A and 5-HT2 receptors as mediators of the interaction between habenula kisspeptin and the serotonin system in the modulation of fear response (Nathan *et al.*, 2015a).

In summary, kisspeptin and its receptor are widely distributed in limbic brain structures and there is mounting evidence that kisspeptin-mediated pathways play crucial roles in sexual behaviour, olfactory processing, mood and emotions. Given its far-reaching actions outside of its classical role in the HPG axis, it is imperative to investigate the effects of kisspeptin on limbic brain function in humans. The first human study of kisspeptin's effects on human brain processing revealed key roles for kisspeptin in sexual and emotional brain processing in response to visual sexual stimuli. Further work is required to explore the effects of kisspeptin on other aspects of human emotions and behaviour.

1.13 Kisspeptin's role in energy intake and metabolism

Energy balance is central to the control of reproduction and the HPG axis can be affected by both malnourishment and over-nutrition. The hypothalamic expression of Kiss1 and Kiss1r is known to respond to both internal and external cues including seasonal variation, sex steroid milieu and metabolic status (Navarro & Tena-Sempere, 2012; Greives *et al.*, 2011; George *et al.*, 2012).

1.13.1 Kisspeptin expression responds to nutritional status

In pre-pubertal rats, short-term fasting reduces expression of Kiss1 and enhances expression of Kiss1r mRNA (Castellano *et al.*, 2005). Whereas short-term fasting in mice leads to decreased expression of both Kiss1 and Kiss1r mRNA (Luque *et al.*, 2007). On the other end of the spectrum, diet-induced obesity in rodents either has no effect on Kiss1 and Kiss1r expression (Luque *et al.*, 2007; Dudek *et al.*, 2016) or reduces expression of Kiss1 and Kiss1r (Zhai *et al.*, 2018). Furthermore, hypothalamic Kiss1 mRNA is also reduced in rat models of

uncontrolled diabetes (Dudek *et al.*, 2016; Castellano *et al.*, 2006). Overall, this suggests that both states of energy restriction and energy excess can attenuate Kiss1 expression. Obesity and diabetes are disorders of energy imbalance so the inability to appropriately utilise high energy reserves in this situation could be a potential reason for reduced Kiss1 expression (Wahab *et al.*, 2013).

In primates, reduced hypothalamic Kiss1 and Kiss1r mRNA expression occurs in fasted male rhesus monkeys compared to fed controls (Wahab *et al.*, 2011) with attenuated HPG responses to exogenous kisspeptin as demonstrated by reduced testosterone concentration, lower testosterone area under the curve and delayed peak in testosterone levels (Wahab *et al.*, 2008). Despite some conflicting findings and species differences, there is clear evidence that kisspeptin is responsive to external cues of nutritional status. However, it is interesting to note that these studies have only examined hypothalamic Kiss1 and Kiss1r expression, yet Kiss1 and Kiss1r are also abundantly distributed in several key brain regions e.g. the amygdala, which communicate with the hypothalamus and are associated with appetite and food-reward behaviours (Berthoud, 2011). Consequently, the question of how extra-hypothalamic kisspeptin signalling interacts with nutritional status remains unaddressed.

1.13.2 Kisspeptin signalling affects body weight and food intake in rodents

Whole body deletion of *Kiss1r* leads to increased adiposity in male and female mice despite reduced food intake (Tolson *et al.*, 2014; Velasco *et al.*, 2019). In terms of body weight, some studies observe that *Kiss1r* knockout females have significantly higher body weight compared to controls whereas *Kiss1r* knockout males either maintain a normal bodyweight (Tolson *et al.*, 2014; Luo *et al.*, 2016) or have lower body weight compared to controls despite increased adiposity and reduced feeding (Velasco *et al.*, 2019). It is possible that some of the perturbations in body weight and body composition can be attributed to hypogonadism in a whole-body *Kiss1r* knockout model. However, differences in adiposity and body weight remain when gonadal status of *Kiss1r* knockout mice is equivalent to controls, either by gonadectomy

of both groups or selective re-expression of *Kiss1r* in GnRH neurons (Tolson *et al.*, 2014; Velasco *et al.*, 2019). Therefore, kisspeptin is likely to exert some of its effects on energy homeostasis independently of the HPG axis through interactions with other neurotransmitters.

1.13.3 Interactions between kisspeptin and appetite-regulating pathways

Given the evidence that kisspeptin may have a role in the regulation of body weight, examination of the relationships between kisspeptin and central appetite-regulating pathways are crucial. Within the arcuate nucleus of the hypothalamus, two distinct neuronal populations act to coordinate energy balance. Agouti-related peptide (AgRP) neurons co-express AgRP and neuropeptide Y (NPY) which act to stimulate food intake (orexigenic) and weight gain. Pro-opiomelanocortin/cocaine- and amphetamine-regulated transcript (POMC/CART) neurons co-express POMC and CART, which decrease food intake (anorexigenic) and promote weight loss (Sam *et al.*, 2012). Neuroanatomical connections have been found between hypothalamic *Kiss1* neurons, AgRP- and POMC-positive cells in mice and sheep (Backholer *et al.*, 2010; Nestor *et al.*, 2016). Functionally, *in vitro* experiments carried out on hypothalamic arcuate slices show that kisspeptin administration increases POMC neuronal firing and inhibits NPY neuronal firing (Fu & Van Den Pol, 2010). Mechanistically, the excitatory actions of kisspeptin on POMC neurons are thought to be mediated directly via *Kiss1r* and also through post-synaptic glutamatergic input (Fu and Van Den Pol, 2010; Nestor *et al.*, 2016). NPY neurons express GABA receptors and kisspeptin has been observed to inhibit NPY neurons *in vitro* through an indirect pathway involving GABA-mediated inhibitory synaptic tone (Fu and Van Den Pol, 2010). Equally, reports also suggest that stimulation of AgRP fibres directly inhibits *Kiss1* in the arcuate and AVPV (Padilla *et al.*, 2017). However, *in vivo* studies in sheep show that kisspeptin administration decreases POMC expression (Backholer *et al.*, 2010) and increases NPY expression (Backholer *et al.*, 2010; Foradori *et al.*, 2017). This contradicts findings in desert jerboa and dwarf hamsters in which kisspeptin increases POMC expression (Talbi *et al.*, 2016; Cázares-Márquez *et al.*, 2019).

Kisspeptin is also known to interact with peripheral signals of nutritional status, namely leptin. Mutations in leptin or the leptin receptor in humans are associated with hypogonadism (Farooqi & O'Rahilly, 2009) and while GnRH neurons do not express the leptin receptor, work in mice has shown co-expression of leptin receptor and Kiss1 mRNA in the arcuate nucleus (Smith *et al.*, 2006a). Leptin infusion into leptin-deficient *ob/ob* mice significantly increases Kiss1 and Kiss1r mRNA levels in the hypothalamus (Luque *et al.*, 2007). Additionally, leptin but not insulin or insulin-like growth factor stimulates Kiss1 mRNA expression in the mouse hypothalamic cell line N6 (Luque *et al.*, 2007). Moreover, in male rats with streptozotocin-induced diabetes, central infusion of leptin can normalise hypothalamic Kiss1 mRNA levels as well as LH and testosterone concentrations (Castellano *et al.*, 2006). However, more recent data indicates that selective leptin receptor deletion from hypothalamic Kiss1 neurons has no effect on puberty or fertility in mice and even after the onset of puberty, only a small percentage of Kiss1 neurons express the leptin receptor (Donato Jr *et al.*, 2011). Further work has demonstrated that selective restoration of leptin expression on Kiss1 neurons in leptin receptor knockout mice does not improve the pubertal and fertility deficits seen in these animals (Cravo *et al.*, 2013). These data indicate that direct leptin action on kisspeptin neurons is not an essential mechanism for reproductive maturation. However, leptin-expressing cells in the ventral pre-mammillary nucleus in mice project to Kiss1 neurons, and leptin activity in this area has permissive effects on puberty and fertility, thus leptin may still modulate kisspeptin indirectly via intermediate factors (Donato Jr *et al.*, 2011).

In summary, although kisspeptin may have a role in sensing and translating metabolic conditions into signals for the reproductive axis, the interplay between kisspeptin signalling in reproduction and metabolism is likely to involve several neuro-humoral components that have yet to be fully uncovered (Roa, 2013).

1.14 Effects of kisspeptin on feeding behaviour

The effects of kisspeptin administration on feeding behaviour have been studied in a small number of rodent experiments with some conflicting results. Acute central (ICV) administration of kisspeptin inhibits feeding in a dose-dependent manner in fasted adult male mice (Stengel *et al.*, 2011) and rats (Saito *et al.*, 2019). In mice, ICV kisspeptin significantly decreases meal frequency and total time spent in meals whereas meal size and gastric emptying does not significantly differ from vehicle (Stengel *et al.*, 2011). Furthermore, the effects of ICV kisspeptin administration in male rats is associated with increased expression of the anorexigenic neuropeptides; nesfatin-1 and oxytocin (Saito *et al.*, 2019). However, other studies have shown that central kisspeptin administration does not alter feeding behaviour in *ad libitum* fed male rats (Castellano *et al.*, 2005; Thompson *et al.*, 2004). Furthermore, Stengel *et al* did not find any changes in feeding behaviours when kisspeptin was administered peripherally via a 10µg (0.01µmoles) intraperitoneal injection (Stengel *et al.*, 2011). However, a more recent study utilising a higher dose (10µmoles) of peripheral kisspeptin elicited significant reduction in food intake, meal frequency, meal size and eating rate in *ad libitum* fed adult male and female mice (Dong *et al.*, 2019).

Taken together, these studies suggest that exogenous kisspeptin administration can moderate food intake in male and female rodents, which adds to the increasing body of animal evidence for a central role of kisspeptin in appetite-regulating pathways. There is now a clear need to explore the role of kisspeptin in human appetite and food-related behaviours, however translational studies in this area have yet to be performed.

1.15 Summary

The neuropeptide kisspeptin is now established as a master regulator of the reproductive axis. However, ever since early reports, expression of kisspeptin and its receptor have been observed in multiple tissues outside the classical hypothalamic reproductive circuit (Lee *et al.*,

1999; Kotani *et al.*, 2001; Muir *et al.*, 2001; Gottsch *et al.*, 2004; Clarkson *et al.*, 2009; Herbison *et al.*, 2010). In recent years, a rapidly expanding body of animal literature has emerged to help us understand the functional roles of these extra-hypothalamic kisspeptin populations. In doing so, a complex network of afferent and efferent connections as well as excitatory and inhibitory signals have been uncovered to provide a functional and neuroanatomical framework through which kisspeptin-signalling may integrate multiple internal and external cues (including visual, olfactory, and nutritional signals) with appropriate behavioural and neuroendocrine responses (Comninou and Dhillo, 2017).

Translating these findings into humans is of paramount importance as very little is currently known regarding human psychosexual function, disorders of which affect up to one third of individuals worldwide (Laumann *et al.*, 1999; Mitchell *et al.*, 2013) with detrimental effects on quality of life and interpersonal relationships (Khademi *et al.*, 2008). To date there has only been one human study investigating the role of kisspeptin in human brain processing. The results show that kisspeptin significantly enhances sexual and emotional brain processing in healthy men in response to sexually stimulating images and this correlates with reduced sexual aversion and reduced negative mood (Comninou *et al.*, 2017). Given that kisspeptin has a central role in mate attraction and olfactory processing (Kauffman *et al.*, 2007; Pineda *et al.*, 2017; Hellier *et al.*, 2018), and olfactory function is closely related to sexual function in humans (Ottaviano *et al.*, 2013; Özmen *et al.*, 2016) further work exploring the role of kisspeptin on human brain responses to attraction cues such as olfactory inputs would be very informative.

Furthermore, kisspeptin has been implicated as an integral link between metabolic signals and the reproductive axis, however until now *in vivo* human studies have not been performed to investigate the effects of kisspeptin on human appetite and feeding behaviour.

1.16 Aims and hypotheses of the thesis

This PhD thesis focusses on exploring the functional roles of kisspeptin in human attraction, appetite and feeding behaviour. To address this, I generated the following hypotheses and aims which I will describe in greater detail in the following chapters:

Chapter 2

Aim: To investigate the effects of peripheral kisspeptin administration on brain responses to olfactory and visual cues of attraction using fMRI, psychometric and hormone analyses in healthy men.

Hypothesis: Peripheral administration of kisspeptin in healthy men enhances brain processing in response to olfactory and visual cues of attraction.

Chapter 3

Aim: To investigate the effects of peripheral kisspeptin administration on appetite and food intake during an *ad libitum* meal.

Hypothesis: Peripheral kisspeptin administration suppresses appetite and food intake in healthy men.

Chapter 4

Aim: To investigate the effects of peripheral kisspeptin administration on brain responses to viewing food images using fMRI, psychometric and hormone analyses in healthy men.

Hypothesis: Peripheral administration of kisspeptin in healthy men modulates brain processing in response to viewing food images.

Chapter 2

The effects of peripheral kisspeptin administration on brain responses to olfactory and visual cues of attraction in healthy men.

2.1 Introduction

Reproduction is essential for preservation of the species, with attraction to another individual serving as the fundamental initiating step for this innate physiological process (Fisher, 1998). Attraction relies on the integration of multimodal social and sensory cues with appropriate emotional and behavioural outputs. However, the intrinsic mediators of human attraction remain incompletely understood. Unravelling these integral processes is of major scientific and clinical importance, as disturbances in psychosexual function are known to affect up to 1 in 3 people worldwide (Laumann *et al.*, 1999; Mitchell *et al.*, 2013), with significant detriments in quality of life, interpersonal relationships and fertility (Khademi *et al.*, 2008). Furthermore, despite the high clinical burden, our understanding of the brain processes governing human psychosexual function remains limited and has restricted the development of effective treatments (Rubio-Aurioles & Bivalacqua, 2013).

In trying to identify the intrinsic factors that integrate and co-ordinate human attraction with limbic and reproductive pathways, the HPG axis may provide a potential candidate. There is increasing evidence suggesting that the HPG axis interacts with other neural systems to modulate sexual behaviour (Moss & McCann, 1973; Rissman *et al.*, 1997; Pasch *et al.*, 2011). Furthermore, hypogonadal states in animals and humans result in loss of responsiveness to sexual stimuli and loss of appetitive sexual behaviours (Boling & Blandau, 1939; Christensen & Clemens, 1974; Matsumoto, 2002). The neuropeptide kisspeptin is a master regulator of reproduction and sits at the apex of the HPG axis where it activates GnRH and downstream hormone release (de Roux *et al.*, 2003; Seminara *et al.*, 2003; Messenger *et al.*, 2005; Tena-Sempere, 2008; Navarro & Tena-Sempere, 2012; Hanchate *et al.*, 2012; Hrabovszky, 2014). Kisspeptin and its receptor are also widely expressed in limbic brain regions (Muir *et al.*, 2001; Kotani *et al.*, 2001; Gottsch *et al.*, 2004; Clarkson *et al.*, 2009) with animal work showing that kisspeptin influences brain pathways controlling male and female reproductive behaviours (Gresham *et al.*, 2016; Adekunbi *et al.*, 2018; Hellier *et al.*, 2018). Furthermore, kisspeptin neurons interconnect olfactory, limbic and hypothalamic reproductive centres in rodents

(Pineda *et al.*, 2017) and kisspeptin activity at its receptor is requisite for olfactory-mediated partner preference (Kauffman *et al.*, 2007; Hellier *et al.*, 2018). Thus, kisspeptin may be a novel factor in human attraction, which has not been investigated until now.

2.1.1 Olfactory cues of attraction in humans

Olfaction underpins many important mating behaviours in mammals, through the detection of chemosignals; volatile substances secreted externally by an individual and received by a second individual of the same species leading to specific physiological reactions (Parkes & Bruce, 1961; Petrulis, 2013). Olfactory signals are central to mate attraction and have been extensively studied in non-human mammals (Yoon *et al.*, 2005). Although human sexual behaviours are more complex, relying on context and learning as well as innate behaviours, evidence suggests that olfaction still plays a significant role in our sexual responses. For example, men exhibit increased sexual attraction towards odours from women in the ovulatory phase compared to odours from women in the luteal phase (Gildersleeve *et al.*, 2012). There is also a significant correlation between olfactory function and psychosexual function in human males (Ottaviano *et al.*, 2013; Özmen *et al.*, 2016) with reported improvements in sexual function following treatment of chronic nasal disorders (Gunhan *et al.*, 2011). The human primary olfactory network has numerous projections to key limbic areas involved in sexual and emotional processing (Fjaeldstad *et al.*, 2017). Furthermore, when heterosexual men are exposed to a female scent, they exhibit increased brain activity on functional neuroimaging in limbic regions associated with sexual desire and arousal (Huh *et al.*, 2008). Taken together, these data suggest that olfactory signals are significant cues of attraction in humans and studying factors that modulate brain responses to olfactory cues could provide important insights into human psychosexual function.

2.1.2 Visual cues of attraction in humans

The visual appreciation of beauty is another fundamental aspect of human attraction with evidence that prefrontal areas, in particular the medial pre-frontal cortex, are heavily involved in the human perception of beauty (O'Doherty *et al.*, 2003; Cela-Conde *et al.*, 2004; Ishai, 2007; Winston *et al.*, 2007). Facial beauty provides a symbol of sexual potential and reproductive fitness, with evidence that men show consistent cross-cultural preferences for certain female facial characteristics such as symmetry and feminised features including a small nose and narrow jawline (Perrett *et al.*, 1998; Penton-Voak *et al.*, 1999). Thus, studying factors that may modulate brain responses to visual cues of attraction could provide important insights into human psychosexual function.

2.2 Principles of functional magnetic resonance imaging (fMRI)

Functional magnetic resonance imaging (fMRI) is a non-invasive technique used to assess changes in brain activity through measurement of blood oxygen level dependent (BOLD) activity in the brain. The BOLD signal is derived from the concept that deoxygenated and oxygenated haemoglobin have different magnetic susceptibility properties (Ogawa *et al.*, 1990). Active neurons receive oxygenated blood at a greater rate than (comparatively) inactive neurons leading to a local change in the ratio of oxygenated and deoxygenated haemoglobin. This intrinsic MRI contrast can then be visualised as a map of brain activation with the magnitude of the BOLD signal serving as a surrogate measure of neuronal activity (Friston *et al.*, 1994).

2.2.1 Task – based fMRI

Task-based MRI is an established practice that investigates differential neuronal responses to various stimuli and activity during task performance (Heeger & Ress, 2002). The use of tasks selects out specific brain pathways of interest from background brain activity that occurs at

rest. When an additional intervention is applied (e.g. a pharmacological probe, or a hormone infusion) the results from task-based fMRI represent an interaction effect between the selected tasks (which target pathways of interest i.e. olfactory and visual attraction networks), and the effects of the intervention e.g. kisspeptin administration versus vehicle administration.

Task design is an important first step for any fMRI study. Two main types of task design are commonly employed; block-design and event-related designs. The choice of design depends on the type and delivery of the stimulus and affects the choice of subsequent statistical modelling.

Block design

This comprises a series of long blocks that last between 10 – 60 seconds, where the participant is asked to perform the same mental process for the full duration of the block (e.g. viewing and rating pleasant images) (Buckner et al., 1996). Typically, blocks of active task performance are interspersed with resting blocks, which are used as a baseline for statistical analysis. Block-design tasks are the simplest to construct and analyse since similar trials are blocked and modelled together. However, this design is not suitable for tasks that cannot be blocked (e.g. where some random presentation of task conditions is desirable), or for separating the BOLD response for trials within a block. In addition, block-designs involve repetition of the same stimulus-type, which could lead to habituation or a change in the way participants process the task information resulting in a difference in response due to familiarity, fatigue and/or boredom. Nonetheless, within the constraints noted above, this design is the most powerful, sensitive, and efficient design for fMRI task paradigms (Amaro & Barker, 2006).

Event-related design

This design consists of trials presented for a shorter period of time (e.g. 1 - 5 seconds) with different types of trials presented in a random order (Friston *et al.*, 1998). For example, to design a task looking at brain responses to pleasant, neutral and aversive images, a block-

design would involve grouping these images into three block types, with images presented sequentially within each block, interspersed with a rest period (e.g. a blank screen). An event-related design would present the participant with a continuous series of pleasant, neutral and aversive images in a random order. Each image trial is separated by a varying time delay called the inter-trial interval (ITI), usually lasting 2-15 seconds. This randomisation or 'jitter' of the ITIs is designed to create a response profile with a complex waveform that can be better distinguished from the more regular background noise waveforms. Similar to rest periods in block-design tasks, the ITI periods provide a baseline for statistical analyses in event-related designs (Dale, 1999). Event-related designs are more flexible than block-designs and remove the anticipatory and habituation effects inherent within block-design tasks. They are therefore well-suited to most standard psychological/cognitive tasks. Furthermore, event-related designs usually require participants to switch their attention between different types of stimuli, which contributes to a more alert state during the task. However, a disadvantage of event-related designs is that they often require more trials, and are generally less powerful from a signal-detection perspective (Amaro & Barker, 2006). This results in longer tasks and generates a cognitive and attentional load that may lead to fatigue in participants. In this present thesis both design types have been utilised for different fMRI tasks depending on the specific hypothesis proposed.

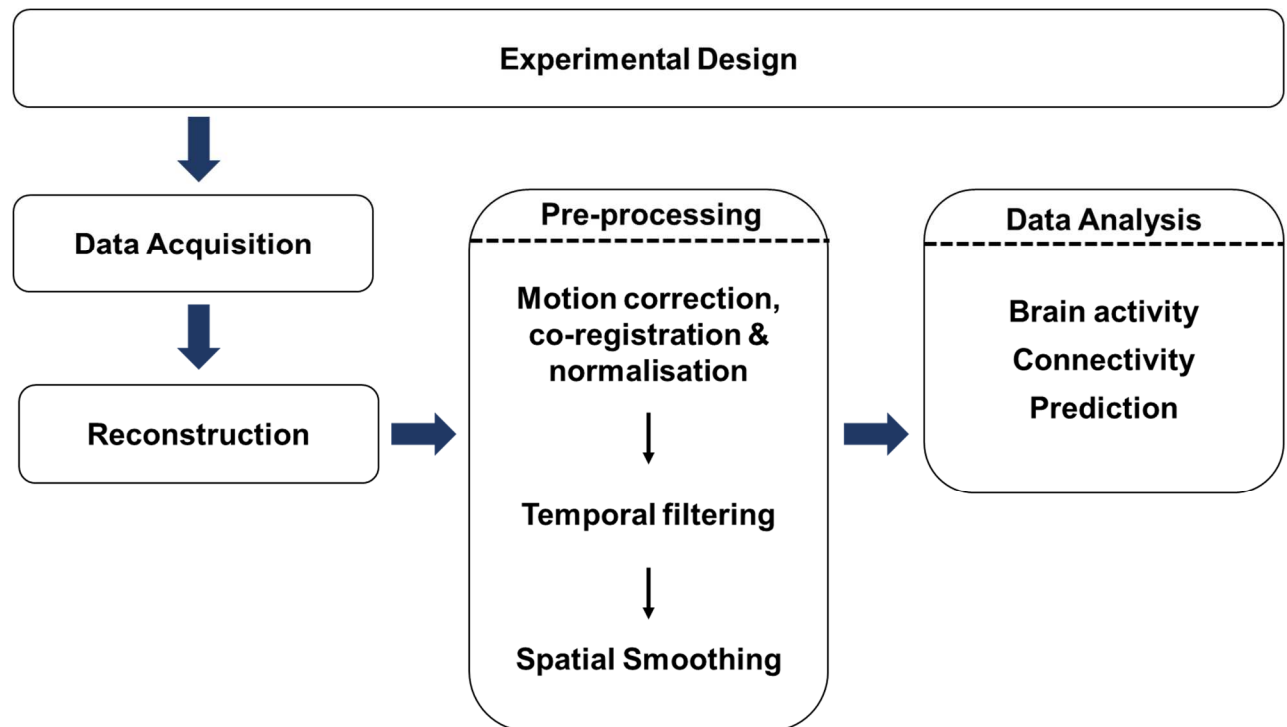
2.2.2 Overview of fMRI data acquisition, processing and analysis

Following experimental design, fMRI data must be taken through a series of processes from data acquisition through pre-processing to analysis (see Figure 2.1 for a schematic overview). Acquired fMRI data is reconstructed into images and data from each task is pre-processed separately. Firstly, the anatomical image is skull-stripped, and any non-brain tissue is removed to extract the brain. A series of pre-processing steps correct for artefacts due to the susceptibility to noise caused by head movements, scanner drift, or other brain processes not related to the specific experimental task. In general, pre-processing includes head motion

correction which re-aligns the functional scans across time, and spatial smoothing to improve the signal-to-noise ratio by replacing the BOLD value of each voxel with a weighted average of the neighbouring voxels. Additionally, the registration process is carried out, which aligns the functional scan of an individual to the same person's structural (anatomical) scan and then to a standard stereotactic space, allowing easier visualisation and interpretation of the results at the next stage (Soares *et al.*, 2016).

Statistical analysis occurs at a first-level for each individual and then at a second level for the group of participants as a whole. During the first-level, an application of the General Linear Model is used (Pernet, 2014). This produces an informed model of the BOLD response to each stimulus, which is applied to every unit of measurement ('voxel') in the brain individually. Other regressors may be added to the model as regressors of no-interest or confound regressors e.g. head-motion regressors and regressors derived from physiological data (e.g. respiration, heart rate). The purpose of these regressors is to produce a more accurate model of the responses, and therefore to reduce the overall residual error. Contrasts are specified in each first-level model, typically these will be comparisons between experimental conditions, and/or comparisons of experimental conditions against baseline (rest blocks or ITI periods). The regression coefficients (beta-weights) for the contrasts of interest from each first-level model are then entered into a higher-level analysis where group-level confounds (e.g. visit order, age, sex) can also be entered, a statistical threshold is chosen, and the results are corrected for multiple comparisons, because of the large number (typically >100,000) of statistical tests performed. Many schemes for multiple comparison correction exist, but the most commonly-used one involves applying an initial threshold in order to define clusters of activation, and then a further statistical correction based on the number of active clusters (Woo *et al.*, 2014).

Figure 2.1: Data processing pipeline for a fMRI study



Following experimental design, data acquisition is performed, followed by reconstruction of the data into images. Pre-processing addresses issues such as anatomical alignment of various image types (*co-registration*), head movement (*motion correction*), filtering of low-frequency noise signals (*temporal filtering*), and image transformation onto a standard anatomical reference space (*spatial smoothing, normalisation or warping*). After pre-processing, the images are ready for statistical analysis. Analyses can test task- or outcome-related brain activity, assess functional connectivity, and develop multivariate predictive models designed to correlate optimally with experimental variables or outcomes.

2.2.3 Advantages and drawbacks of fMRI

fMRI is a very objective measure of brain response to a stimulus as it does not rely on subjective answers to behavioural tasks or questionnaires. It gives information on the brain mechanisms that are in place to process complex sensory and emotional inputs and it also allows brain activation to be linked to functional behaviour. Moreover, fMRI has higher spatial resolution compared to other non-invasive functional imaging techniques such as positron emission tomography and electroencephalography (Ogawa *et al.*, 1990). This, along with good temporal resolution, reasonable signal-to-noise ratio, and the ability to reliably image deep brain structures including the hippocampus, amygdala and the striatum has made fMRI the current preferred method for most cognitive neuroscience applications. In addition, fMRI is a highly flexible technique in terms of stimulus presentation and experimental design, with the ability to use various stimuli e.g. visual, olfactory, auditory, pain and taste. This makes fMRI ideal for assessing an array of cognitive and emotional modalities (Logothetis, 2008).

In terms of disadvantages, BOLD signal is not a direct measure of neuronal activity, however neurovascular coupling studies using implanted brain electrodes to record electrophysiology in awake monkeys have shown that BOLD signal generally correlates very closely with neuronal activity, especially post-synaptic neuronal activity (Goense & Logothetis, 2008). There is also a lag time of 2 - 5 seconds after a stimulus before BOLD activity peaks and there may be some variation depending on cortical region (Schleim & Roiser, 2009), however current fMRI analysis software such as FSL (Oxford, UK) are designed to take this into account.

2.3 Aims and hypothesis

Aims:

- To investigate the effects of peripheral kisspeptin administration compared to vehicle on human brain processing during olfactory and facial attractiveness tasks by measuring changes in fMRI BOLD activity.
- To correlate kisspeptin-related changes in fMRI BOLD activity with psychometric parameters and hormone profiles.

Hypothesis: Kisspeptin administration in healthy men can enhance brain processing in response to olfactory and visual cues of attraction.

2.4 Methods

2.4.1 Study approval

The study was performed in accordance with the Declaration of Helsinki. All participants gave written informed consent prior to inclusion in the study. The study was approved by the regional ethics committee (Riverside Research Ethics Committee, London, United Kingdom, REC ref: 17/LO/1504).

2.4.2 Sample size

Data from previous work in my lab (Comninos *et al.*, 2017) demonstrates that kisspeptin enhanced task-based brain activity, measured by BOLD signal change in the amygdala by mean 0.74% and standard deviation 0.38% compared to vehicle (mean 0.48%, standard deviation 0.51%) during a fMRI task, and we anticipated a similar response in this study. Using these data, with power of 80%, significance level (alpha) of 5% and assuming correlation between means of 0.40, a power calculation was performed resulting in a sample size of 31. This is in line with previous fMRI studies and empirically-derived estimates of optimal sample sizes in fMRI studies (Murphy & Garavan, 2004; De Silva *et al.*, 2011; Comninos *et al.*, 2017).

2.4.3 Study recruitment and eligibility criteria

To achieve the sample size while allowing for drop-outs and exclusion rates (of 15%, from previous experience), a total of 36 participants were recruited into the study via print and online advertisements. To confirm eligibility, participants attended a medical screening appointment. Participants were free of current and past physical or psychiatric illness and were naive to psychoactive substances, prescribed or illicit. The Patient Health Questionnaire-9 was used to screen for depression and those scoring above threshold for depressive disorder were excluded from the study (Kroenke *et al.*, 2001). Heterosexuality was determined by a Kinsey score of zero (Kinsey *et al.*, 2003), and abnormal olfactory function was screened out by the

Brief Smell Identification Test (Doty *et al.*, 1996). Specific assessment was also undertaken to exclude participants with any history of sexual aggression/abuse/phobia or psychotherapy/counselling. In addition, tobacco smokers were excluded as smoking is associated with olfactory deficits that could interfere with the olfactory fMRI task (Katotomichelakis *et al.*, 2007). All participants were right-handed and had normal or corrected-to-normal vision. Full inclusion and exclusion criteria are shown in Table 2.1.

Table 2.1: Inclusion and exclusion criteria

Inclusion Criteria
Males aged 18 – 36 years
Right-handed
Non-smoker
Heterosexual (Kinsey Score = 0)
Normal olfactory function
Normal basal reproductive hormone levels
Normal resting electrocardiogram
Exclusion Criteria
Any significant current or past physical or psychiatric illness
History of psychotherapy or counselling
Use of psychoactive substances prescribed or illicit in the 6 months prior to screening visit
History of sexual aggression/abuse/phobia
Any contraindications for MRI scanning as listed in MRI safety guidelines (Appendix 1)

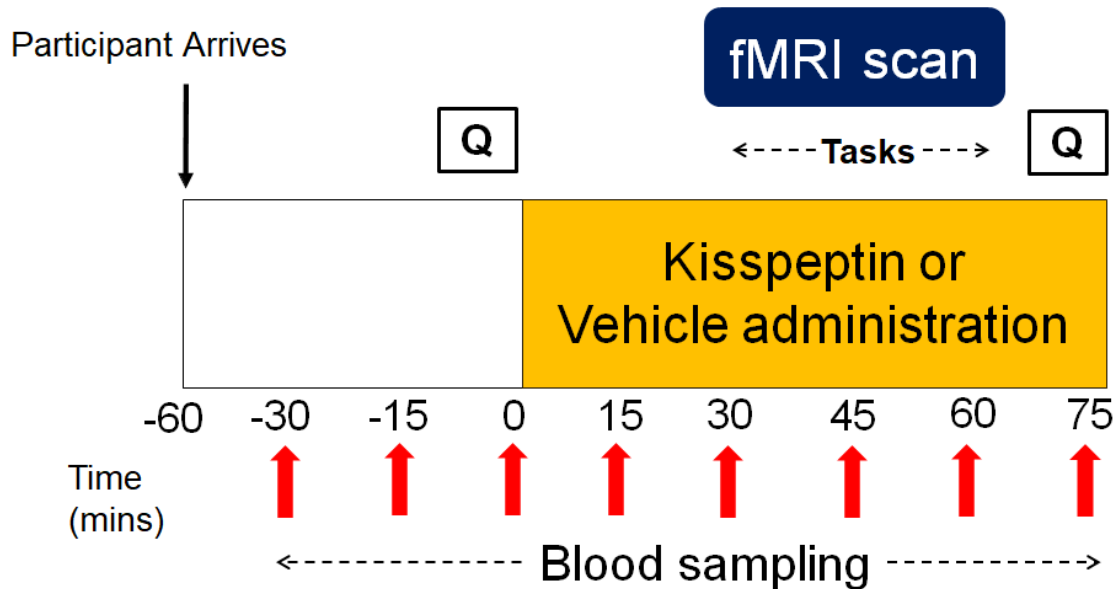
2.4.4 Study day protocol

Participants completed two MRI study visits each and were randomised by an independent investigator (using Research Randomizer, www.randomizer.org) to receive either kisspeptin-54 or vehicle at their first visit (Figure 2.2). This was a within-participant design study, in which the participants acted as their own control thereby minimising variability and enhancing power. All study visits commenced in the morning to control for circadian hormonal changes. Participants were asked to abstain from sexual activities from midnight before the study visit, since prior sexual activity can affect testosterone levels (Shulman & Spritzer, 2014; Fox *et al.*, 1972). In addition, participants were asked to abstain from alcohol and caffeine for the same period of time and to consume a normal breakfast on the morning of their study visit.

On arrival, participants were asked to remove any metal on their person and change into loose hospital scrubs. Intravenous cannulae were inserted into each antecubital fossa for blood collection and infusion of kisspeptin or vehicle. Blood sampling took place at 15 minute intervals from -30 to 75 minutes to measure circulating kisspeptin, LH, testosterone and cortisol levels and to ensure that baseline reproductive hormone levels were equivalent between study visits. At T = 0 minutes, a 75 minute infusion of kisspeptin-54 (1nmol/kg/hour) or vehicle (gelofusine at equivalent rate) was given. This kisspeptin dose was selected based on previous experience (Comninou 2017), to ensure steady-state levels of circulating kisspeptin from 30 to 75 minutes (during fMRI scanning and tasks) while avoiding increase in downstream testosterone during this time-frame.

Participants completed psychometric questionnaires before and at the end of the kisspeptin or vehicle infusion as detailed below. fMRI tasks were initiated at 30 minutes from start of infusion to allow plasma kisspeptin levels to reach steady state.

Figure 2.2: Study day protocol



Participants attended for two study visits each, one for intravenous administration of kisspeptin (1 nmol/kg/hour) and one for intravenous administration of an equivalent volume of vehicle for 75 minutes. Participants completed baseline and intra-infusion questionnaires (Q) and blood sampling took place at time-points -30, -15, 0, 15, 30, 45, 60, and 75 minutes to measure circulating kisspeptin, LH, testosterone and cortisol during the study. Infusion commenced at T = 0 minutes. At T = 30 minutes when kisspeptin levels reached steady state, participants underwent functional MRI scanning while performing visual and olfactory tasks.

2.4.5 Kisspeptin-54 peptide preparation

Human sequence kisspeptin-54 peptide was synthesised by Bachem (St Helens, UK). Purification was performed by reverse-phase High Performance Liquid Chromatography. Electrospray mass spectroscopy and amino acid analysis confirmed identity of the peptide. Toxicology testing in animals was conducted prior to administration to human volunteers. The limulus amoebocyte lysate test detected no endotoxin (Associates of Cape Cod, Liverpool, UK), and bacterial culture was sterile (Department of Microbiology, Hammersmith Hospital, London, UK), in samples of kisspeptin-54 peptide. Vials of kisspeptin-54 were freeze-dried and stored at -20°C. Vials were defrosted on the morning of the study and reconstituted in 0.9% saline prior to administration.

2.4.6 Blood sample collection

Blood samples were collected at the timepoints shown in Figure 2.2. Samples for kisspeptin measurement were collected into lithium heparin tubes containing 5000 kallikrein inhibitor units of aprotinin (200µl Trasylol, Bayer) to limit enzymatic breakdown of kisspeptin. Immediately after collection, blood samples were centrifuged at 4000 revolutions per minute (RPM) for 4 minutes at room temperature. Plasma was separated and transferred into 1.5 ml microcentrifuge tubes (Eppendorf). Blood samples for LH, testosterone and cortisol measurement were collected into tubes containing clot activator (BD Vacutainer). After collection, blood samples were left to clot at room temperature for ≥ 30 minutes and then centrifuged at 3000 RPM for 10 minutes at room temperature. Serum was separated and transferred into LP4 tubes. All samples were stored immediately at -20°C then defrosted prior to sample analysis.

2.4.7 Kisspeptin radioimmunoassay

Measurement of plasma kisspeptin immunoreactivity was performed using a manual radioimmunoassay (Dhillon *et al.*, 2005) based on a GQ2 antibody raised in sheep immunised

to synthetic human kisspeptin-54 (Advanced Biotechnology Centre, Imperial College London, UK). The antibody cross-reacts 100% with human kisspeptin-54, kisspeptin-14 and kisspeptin-10 and < 0.01% with other RF amide related peptides (Dhillon *et al.*, 2005). The ¹²⁵I-kisspeptin-54 label was prepared using the iodogen method and purified by reverse-phase High Performance Liquid Chromatography (Salacinski *et al.*, 1981). The assay was performed in duplicate using dilutions of neat plasma in 0.06 M phosphate buffer (pH 7.2) containing 0.3% Bovine Serum Albumin. Set concentrations of pure peptide were also assayed to produce a standard curve for the assay. Samples were incubated for three days at 4°C. Subsequently, free and antibody - bound label were separated by charcoal adsorption. As is standard practice in radioimmunoassays, the proportion of radiolabel present in each fraction enabled direct measurement of the amount of unlabelled antigen that was bound to the antibody by directly referencing the standard curve produced at the same time. The limit of detectability of the assay was 2 pmol/L of plasma kisspeptin with 95% confidence interval, and the intra- and inter-assay coefficients of variation were 8.3 and 10.2%, respectively.

2.4.8 Measurement of circulating hormones

Circulating LH, testosterone and cortisol levels were measured using automated chemiluminescent immunoassays (Abbott Diagnostics, Maidenhead, UK). The intra-assay and inter-assay coefficients of variation were < 5% for LH, and testosterone and < 10% for cortisol. Analytical sensitivities were: 0.5 IU/L (LH), 2 nmol/L (testosterone); 28 nmol/L (cortisol). Limits of detectability for each assay were: LH, 0.07 IU/L; testosterone, 4.9 nmol/L; cortisol, 22 nmol/L.

2.4.9 fMRI procedure

During the MRI session, a series of anatomical and functional brain scans were performed. For the functional tasks, a mirror mounted on the head coil was used to view a screen at the rear of the scanner bore onto which the stimulus images were projected. To respond to the

tasks, the participants were equipped with a custom-made five button MRI-compatible response box. In addition, a pulse-oximeter and a respiratory belt were used to monitor physiological data by means of a standard data-recording system (AD Instruments PowerLab) in the control room. Kisspeptin and vehicle infusions were administered via a Medrad Spectris Solaris MRI compatible injection system controlled from a remote panel in the control room.

2.4.10 Olfactory task

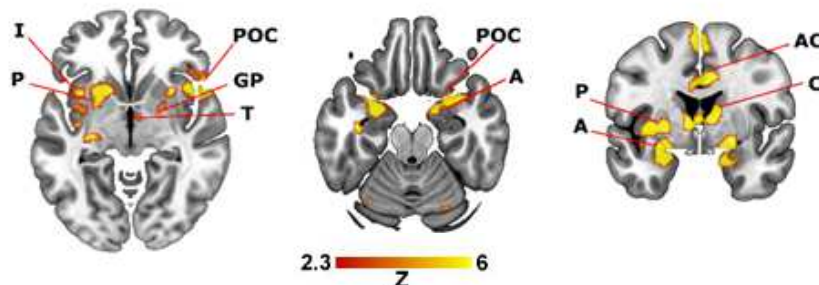
Chanel No.5 was chosen as a recognised feminine scent for the olfactory stimulus as it has been shown in a previous fMRI study to activate limbic regions including the amygdala and thalamus, which are associated with sexual arousal (Huh *et al.*, 2008). During the screening visit, I also blinded participants to the identity of the scent and asked them to rate the scent according to pleasantness and femininity. All participants in the study validated Chanel No.5 as a pleasant and feminine scent at baseline (Table 2.2). Furthermore, I performed a pilot study in five healthy young men and my results showed that Chanel No.5 produced significant activation in limbic structures, similar to the areas modulated by kisspeptin in men viewing sexual images (Figure 2.3). These data confirm that Chanel No.5 would be an appropriate stimulus to examine how kisspeptin modulates limbic brain responses to a feminine odour cue.

As this task involved one type of stimulus only, a block-design was used. During the task, participants received 20 blocks of stimulus comprising nasal delivery of Chanel No.5 (concentration as per manufacturer) for 6 seconds followed by 20 seconds of odourless air as baseline. This protocol was designed based on previously validated olfactory fMRI protocols (Karunanayaka *et al.*, 2014; Wang *et al.*, 2014). The short duration of scent presentation followed by a long period (20 seconds) of odourless air provided enough time to wash out the scent between trials thus limiting olfactory habituation (Wang *et al.*, 2014). To ensure the participants remained alert, a star appeared on the screen at random times during the task and the participants were instructed to respond by pressing a button with their index finger on

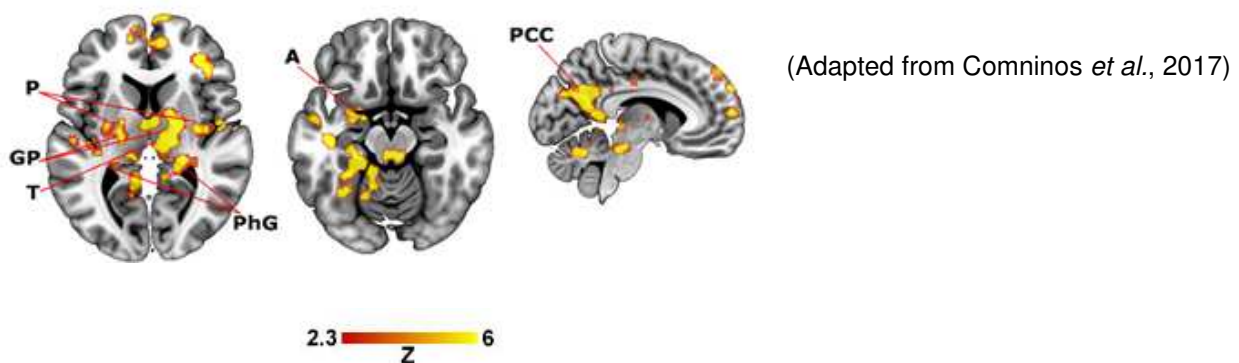
the response box. An ETT1 olfactometer (Emerging Tech Trans) was used to deliver the scent during the olfactory task. The olfactometer was connected to an odorant carrier in the MR suite which transported the scent to the participants via a length of Teflon tubing connected to nasal prongs worn by the participants. This allowed the olfactory stimulus to be delivered at a constant rate to the nasal passage.

Figure 2.3: Pilot study using Chanel No.5 and comparison with brain activity enhanced by kisspeptin

A: Pilot data (N = 5) of brain response to Chanel No.5



B: Kisspeptin enhances limbic brain activity in humans in response to sexually stimulating images



A: Pilot fMRI study in healthy men (n=5) using Chanel No.5 as an olfactory stimulus alternating with air flush. Highlighted areas represent increased brain activity specifically induced by Chanel No.5.

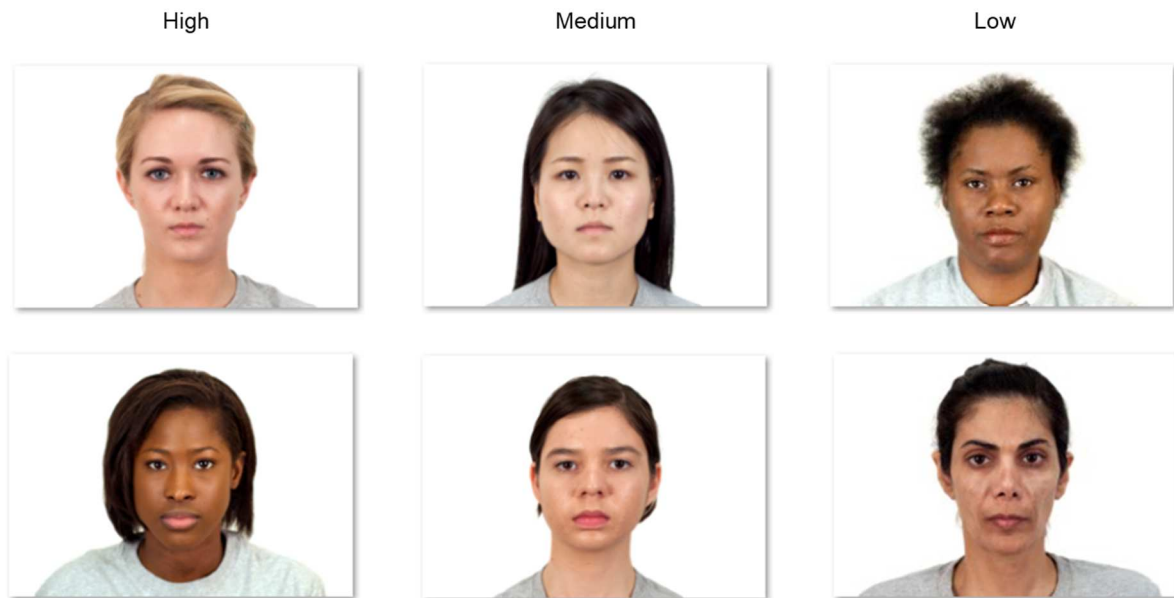
B: Data from an fMRI study in healthy men given kisspeptin vs vehicle. Highlighted areas represent increase in brain activity specifically enhanced by kisspeptin (compared to vehicle) in response to sexually stimulating images (Comninou *et al.*, 2017).

A = Amygdala; AC =Anterior Cingulate; C=Caudate; GP = Globus Pallidus; I=Insula; P = Putamen; PCC = Posterior Cingulate Cortex; PhG = Parahippocampal Gyrus; POC=Primary Olfactory Cortex; T= Thalamus.

Facial attractiveness task

In order to investigate kisspeptin's effects on brain responses to varying levels of facial attractiveness, male participants were presented with images of female faces from the validated Chicago Faces Database (Ma *et al.*, 2015). The database consists of 306 colour photographs of female subjects with forward-facing neutral expressions under standardised lighting. Female subjects were aged between 18 and 40 years and from a range of ethnicities (Caucasian, Asian, Hispanic and African/Caribbean). The database has been independently validated using norming data from 1087 subjective raters who scored each face according to various qualities including attractiveness. Based on these data, 60 female faces were selected for the task (Figure 2.4) comprising 20 faces rated most attractive, 20 faces with medium attractiveness scores and 20 faces with lowest attractiveness scores (Ma *et al.*, 2015). Changes in brain activity in response to viewing faces of high, medium and low attractiveness were compared between kisspeptin and vehicle visits with each participant acting as his own control. This task involved the assessment of three types of stimuli (high, medium and low attractiveness faces), therefore an event-related design was employed. During the task, faces were presented in random order with each face shown once for 4 seconds followed by a jittered inter-trial interval (ITI) of 2 to 10 seconds. The ITI distribution was based on an approximation of a Poisson distribution, as recommended by Hagberg *et al* (Hagberg *et al.*, 2001). To ensure alertness the participants were asked to rate the attractiveness of each face on a five-point scale ranging from "very unattractive" to "very attractive" using the five-button response box.

Figure 2.4: Female faces from the Chicago Face Database



A sample of female faces included in the facial attractiveness task. These were selected from the Chicago Face Database according to their attractiveness rating and grouped into high, medium and low facial attractiveness.

2.4.11 fMRI data acquisition

A 3T Siemens Trio scanner with a 32-channel phased-array head coil was used to acquire functional imaging data. Structural MRI images were acquired at the beginning of each scan using a T1-weighted MPRAGE pulse sequence (1mm isotropic voxels, repetition time (TR) = 2,300ms, echo time (TE) = 2.98ms, flip angle = 9°). Structural images for each participant were reviewed and formally reported by a consultant neuroradiologist. In the event of an incidental finding, I contacted the study participant to discuss the results and wrote a letter to their GP for further investigation and management.

For the acquisition of functional images, a multiband sequence with acceleration factor two was used with the following parameters for the facial attractiveness task: 3mm voxels, TR = 1s, TE = 30ms, flip angle = 80°, 36 axial slices; and for the olfactory task: 2mm voxels, TR = 1.5s, TE = 30ms, flip angle = 80°, 54 axial slices.

2.4.12 fMRI image analysis

FSL software (FMRIB, Oxford) was used to perform fMRI image analysis. Firstly, pre-processing took place, which included motion correction, smoothing (6mm), registration to a standard template (MNI152), and high-pass filtering (0.01Hz). A general linear model analysis modelled the occurrence of the stimuli and included their temporal derivatives and head-motion regressors as confounds. Group analyses were random-effects models, with statistical maps thresholded at $z = 2.3$, $p < 0.05$ (cluster-corrected).

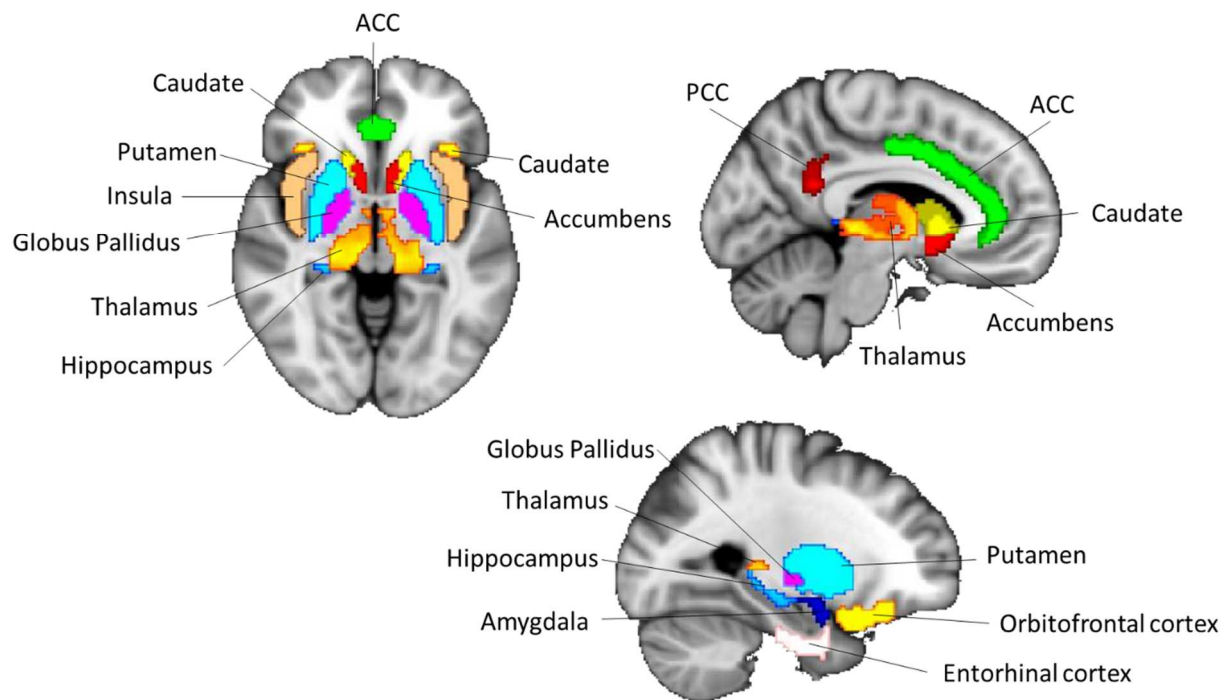
2.4.13 Selecting regions of interest

A set of *a priori* selected brain regions defined in standard stereotactic space using the Harvard-Oxford atlases (<https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/>), was used to extract data for regions of interest (ROI) analyses. For the olfactory task, ROIs were selected based on brain areas expressing kisspeptin receptors in humans (Muir *et al.*, 2001), brain areas involved in

sexual processing (Ferretti *et al.*, 2005) and brain regions that are essential components of the olfactory pathway (Fjaeldstad *et al.*, 2017). This set consisted of the following regions: accumbens, amygdala, anterior cingulate cortex (ACC), caudate, entorhinal cortex, globus pallidus, hippocampus, insula, orbitofrontal cortex (OFC), posterior cingulate cortex (PCC), putamen and thalamus (Figure 2.5). Subsequently, to explore systems-based effects of kisspeptin on olfaction and reproduction, ROI analyses were performed using olfactory and sexual arousal brain masks derived from meta-analytic data within the Neurosynth database (<http://neurosynth.org/>). A motor cortex mask was used as a control.

For the facial attractiveness task, the medial pre-frontal cortex (mPFC) and superior frontal gyrus (SFG) masks were selected as ROIs and functionally defined based on the group mean results across all participants and both conditions (kisspeptin and vehicle). In addition, another ROI analysis was performed based on *a priori* defined brain regions comprising areas known to express kisspeptin receptors and areas involved in sexual arousal (accumbens, amygdala, ACC, caudate, globus pallidus, hippocampus, PCC, putamen and thalamus) (Muir *et al.*, 2001; Comninou *et al.*, 2017).

Figure 2.5 *A priori* anatomically-defined regions of interest for the olfactory task



Anatomically-defined regions expressing kisspeptin receptors and involved in olfactory and sexual processing were derived from the Harvard-Oxford cortical and sub-cortical atlases. For display purposes, the masks are overlaid on the MNI152 stereotactic template brain.

2.4.14 Psychometric questionnaires

Trait questionnaires

At their first study visit, participants were asked to complete a set of psychometric questionnaires to assess certain baseline traits prior to commencing the infusion and starting the MRI scan (Appendix 2). The 20-item State-Trait Anxiety Inventory (Okun *et al.*, 1996) was used to record longstanding trait anxiety levels. The Behavioral Inhibition and Activation System Scale (BIS/BAS) employs a 24-item questionnaire and was used to assess tendency towards anticipation of punishment (BIS) and to reward (BAS) (Carver C.S, 1994). Subjective perceptions of happiness and general satisfaction with life were measured using the Subjective Happiness Scale (Lyubomirsky & Lepper, 1999) and the Satisfaction with Life Scale (Diener *et al.*, 1985) respectively. Additionally, baseline sexual quality of life was assessed using the 11-item Sexual Quality of Life Questionnaire (SQOL) (Abraham *et al.*, 2008). The 14-item Sexual Desire Inventory formally assessed frequency and intensity of desire in normative circumstances of both dyadic (with partner) and solitary sexual desire (Spector *et al.*, 1996). The International Index of Erectile Function is a validated 15-item tool which was used to screen for the five domains of male sexuality (desire, erectile function, intercourse satisfaction, orgasmic function, and overall satisfaction) (Rosen *et al.*, 1997).

State questionnaires

A second set of questionnaires were used to assess emotional state and level of sexual desire in the current moment (Appendix 3). These included the Profile of Mood States short form for adults (POMS2A), a 37-item questionnaire validated for the assessment of psychological distress using the domains of fatigue, vigour, anxiety, anger, depression, confusion and friendliness (Curran *et al.*, 1995). Additionally participants completed the Sexual Arousal and Desire Inventory (SADI), a 55-item multi-dimensional scale to evaluate physiological, emotional, and aversive or inhibitory components within the subjective experience of sexual desire and arousal (Toledano & Pfaus, 2011). Each participant was asked to complete these

questionnaires before and during the end of infusion (kisspeptin or vehicle) on their first and second study visits i.e. each questionnaire was completed four times per participant in total.

2.4.15 Statistical analyses

Statistical analyses were performed using GraphPad Prism 8 software. Data were normally distributed by Kolmogorov testing. Hormone level data analysis was performed using two-way repeated measures analysis of variance (ANOVA) with mixed effects modelling. Individual paired two-tailed t-tests were performed to investigate the kisspeptin effect on each individual ROI. Pearson correlation was used to assess correlations between brain activity and psychometric measures. An α threshold of $p < 0.05$ identified statistical significance in the ROI analyses, but a reduced threshold of $p < 0.01$ was used in the correlation analyses to adjust for the number of analyses performed, in line with previous work (Comninou *et al.*, 2017).

2.5 Results

2.5.1 *Baseline participant characteristics*

In total, 36 participants took part in the study. One participant withdrew, one participant did not complete all the fMRI tasks, and one participant was excluded due to a change in health status during the study, giving a final study group of 33 healthy heterosexual men aged between 18 and 36 years (Table 2.2). Baseline trait questionnaires confirmed that all the participants had normal baseline traits for anxiety, mood, happiness, sexual desire, and erectile function (Table 2.2).

2.5.2 *Effects of kisspeptin administration on circulating hormone levels*

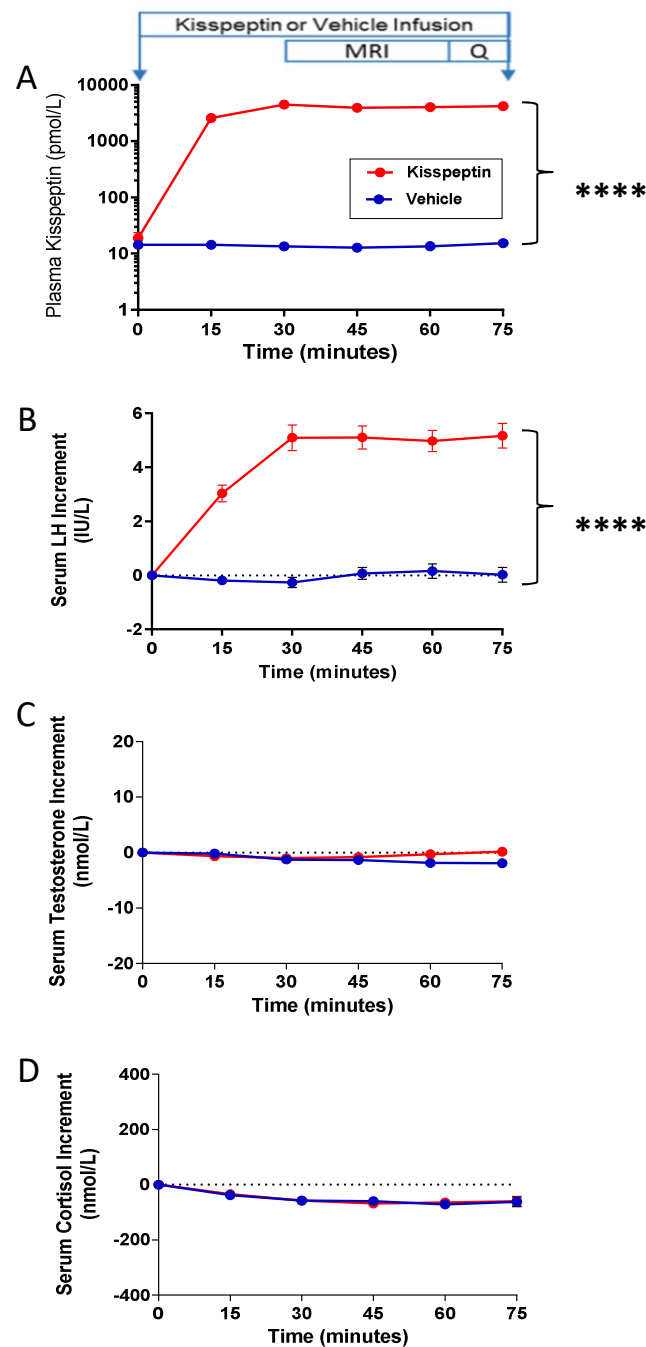
Baseline kisspeptin, LH and testosterone levels were equivalent between study visits (Table 2.3). Subsequently, intravenous kisspeptin infusion (1 nmol/kg/hour) significantly increased circulating kisspeptin levels, reaching steady-state for the duration of fMRI scanning and psychometric questionnaires (Figure 2.6A). As expected, kisspeptin administration led to raised LH levels (Figure 2.6B) which indicated that the dose of kisspeptin used was biologically active. Kisspeptin administration had no significant effects on testosterone levels during the 75 minute study period, as each study was completed before any downstream increases in testosterone (Figure 2.6C), which have previously been shown to occur following longer periods of kisspeptin exposure in humans (Jayasena *et al.*, 2011). Baseline cortisol levels were also equivalent between study visits and kisspeptin administration had no significant effects on cortisol levels compared to vehicle administration (Table 2.3 and Figure 2.6C).

Table 2.2: Participant clinical and psychometric characteristics

		Healthy Men (N = 33)
Ethnicity	Caucasian	49%
	Asian	33%
	Afro-Caribbean	12%
	Mixed/Other	6%
		Mean $\pm$ SEM
Age (years)		24.6 $\pm$ 0.7
BMI (kg/m²)		22.9 $\pm$ 0.3
Baseline Reproductive Hormones	Kisspeptin (pmol/L)	17.9 $\pm$ 2.4
	LH (IU/L)	2.9 $\pm$ 0.2
	Testosterone (nmol/L)	20.8 $\pm$ 1.0
	Cortisol	302.5 $\pm$ 13.3
Number of sexual partners in the last year		1.8 $\pm$ 0.3
Frequency of sexual intercourse per month		5.6 $\pm$ 0.9
Hours viewing pornographic material per week		1.1 $\pm$ 0.2
PHQ-9		1.2 $\pm$ 0.2
BIS		19.4 $\pm$ 0.6
BAS	Drive	11.8 $\pm$ 0.3
	Fun	12.4 $\pm$ 0.4
	Reward	17.5 $\pm$ 0.3
IIEF	Erectile Function	26.8 $\pm$ 1.0
	Orgasmic Function	8.7 $\pm$ 0.4
	Sexual Desire	7.8 $\pm$ 0.2
	Intercourse Satisfaction	10.5 $\pm$ 0.8
	Overall Score	8.4 $\pm$ 0.3
STAI-Y Trait		37.1 $\pm$ 1.3
SDI	Dyadic	47.2 $\pm$ 1.5
	Solitary	17.4 $\pm$ 0.9
	Total	68.4 $\pm$ 2.2
SQOL		80.4 $\pm$ 2.4
SwLS		24.8 $\pm$ 0.8
SHS		17.6 $\pm$ 0.4
Baseline Scent Ratings	Pleasant	3.4 $\pm$ 0.1
	Feminine	4.7 $\pm$ 0.2

BMI = Body Mass Index; LH = Luteinizing Hormone; PHQ-9 = Patient Health Questionnaire-9 to screen for depression; BIS = Behavioral Inhibition System Scale to assess sensitivity to anticipation of punishment; BAS = Behavioral Activation System Scale to assess sensitivity to desired goals, fun and reward; IIEF = International Index of Erectile Function to screen for normal male sexual function; STAI-Y Trait = State-Trait Anxiety Inventory to assess trait anxiety; SDI = Sexual Desire Inventory to assess dyadic and solitary sexual desire; SQOL = Sexual Quality of Life score; SwLS = Satisfaction with Life Scale to assess satisfaction with life as a whole; SHS = Subjective Happiness Scale. Baseline scent ratings were scored on a scale of 0 to 5. Pleasantness: 0 = very unpleasant, 5 = very pleasant. Feminine: 0 = masculine scent, 5 = feminine scent. Data presented as mean $\pm$ SEM.

Figure 2.6: Effects of peripheral kisspeptin administration on circulating hormone levels



A: Kisspeptin infusion increased circulating kisspeptin levels ($p < 0.0001$, $N=33$) reaching steady-state at 30 minutes after initiation, with stable circulating kisspeptin levels during the fMRI and intra-infusion psychometric questionnaires. **B:** In parallel, kisspeptin significantly increased circulating levels of LH ($p < 0.0001$, $N = 33$) compared to vehicle. **C** and **D:** Kisspeptin had no effect on circulating testosterone, or cortisol levels. Data depict mean $\pm$ SEM. **** $p < 0.0001$ using two-way repeated measures ANOVA with mixed effects modelling.

Table 2.3: Baseline reproductive hormone levels during kisspeptin and vehicle visits

	Kisspeptin Visit (N = 33)	Vehicle Visit (N = 33)
	Mean $\pm$ SEM	Mean $\pm$ SEM
Baseline reproductive hormones		
Kisspeptin (pmol/L)	21.1 $\pm$ 4.0	14.7 $\pm$ 1.5
LH (IU/L)	2.9 $\pm$ 0.2	3.0 $\pm$ 0.2
Testosterone (nmol/L)	21.3 $\pm$ 1.1	20.2 $\pm$ 1.1
Cortisol (nmol/L)	295.2 $\pm$ 16.1	309.8 $\pm$ 15.2

LH = Luteinising Hormone. N = 17 participants received vehicle and N = 16 received kisspeptin at first visit. No significant difference in parameters between visits assessed by paired two-tailed t-tests. Hormone levels were within the normal expected physiological ranges. Data presented as mean $\pm$ SEM.

2.5.3 State questionnaires during kisspeptin and vehicle visits

Baseline sexual desire and mood state as assessed by the SADI and POM2A questionnaires were equivalent between kisspeptin and vehicle visits (Table 2.4). Additionally, no significant differences were observed when change in SADI and POM2A scores (pre-infusion – infusion score) were compared between kisspeptin and vehicle visits (Table 2.4).

Table 2.4: Baseline and change in psychometric characteristics during kisspeptin and vehicle visits

		Kisspeptin Visit (N = 33)	Vehicle Visit (N = 33)
		Mean $\pm$ SEM	Mean $\pm$ SEM
Baseline SADI scores	Evaluative	27.2 $\pm$ 4.0	28.9 $\pm$ 3.8
	Negative	16.2 $\pm$ 2.8	18.1 $\pm$ 2.8
	Physiological	15.5 $\pm$ 2.7	16.7 $\pm$ 2.6
	Motivational	14.5 $\pm$ 2.6	15.7 $\pm$ 2.3
Baseline POMS2A scores	Mood Disturbance	45.4 $\pm$ 1.3	45.2 $\pm$ 1.4
	Anger-Hostility	40.4 $\pm$ 0.9	41.0 $\pm$ 1.0
	Confusion-Bewilderment	42.4 $\pm$ 1.2	42.2 $\pm$ 0.9
	Depression-Dejection	43.9 $\pm$ 1.0	43.6 $\pm$ 0.8
	Fatigue-Inertia	41.5 $\pm$ 1.3	41.9 $\pm$ 1.5
	Tension-Anxiety	43.5 $\pm$ 1.4	44.4 $\pm$ 1.7
	Vigor-Activity	42.7 $\pm$ 1.5	45.7 $\pm$ 1.9
	Friendliness	47.4 $\pm$ 1.8	50.7 $\pm$ 1.6
Change in POMS2A scores	Evaluative	0.0 $\pm$ 2.9	3.0 $\pm$ 3.2
	Negative	0.8 $\pm$ 1.4	-1.8 $\pm$ 1.8
	Physiological	1.7 $\pm$ 1.4	3.3 $\pm$ 2.0
	Motivational	-0.3 $\pm$ 1.4	-0.1 $\pm$ 1.7
Change in POMS2A scores	Total Mood Disturbance	1.4 $\pm$ 1.3	-0.6 $\pm$ 1.0
	Anger-Hostility	0.7 $\pm$ 0.7	-0.5 $\pm$ 0.4
	Confusion-Bewilderment	1.3 $\pm$ 1.3	0.5 $\pm$ 0.8
	Depression-Dejection	-1.1 $\pm$ 0.5	-0.8 $\pm$ 0.6
	Fatigue-Inertia	3.4 $\pm$ 1.9	1.6 $\pm$ 1.3
	Tension-Anxiety	-3.5 $\pm$ 1.0	-5.6 $\pm$ 1.1
	Vigor-Activity	-3.7 $\pm$ 1.8	-2.1 $\pm$ 2.0
	Friendliness	-4.1 $\pm$ 1.7	-3.5 $\pm$ 1.1

SADI = Sexual Arousal and Desire Inventory, POMS2A = Profile of Mood States 2 for Adults. N = 17 participants received vehicle and N = 16 received kisspeptin at first visit. No significant difference in parameters between visits assessed by paired two-tailed t-tests. Data presented as mean $\pm$ SEM.

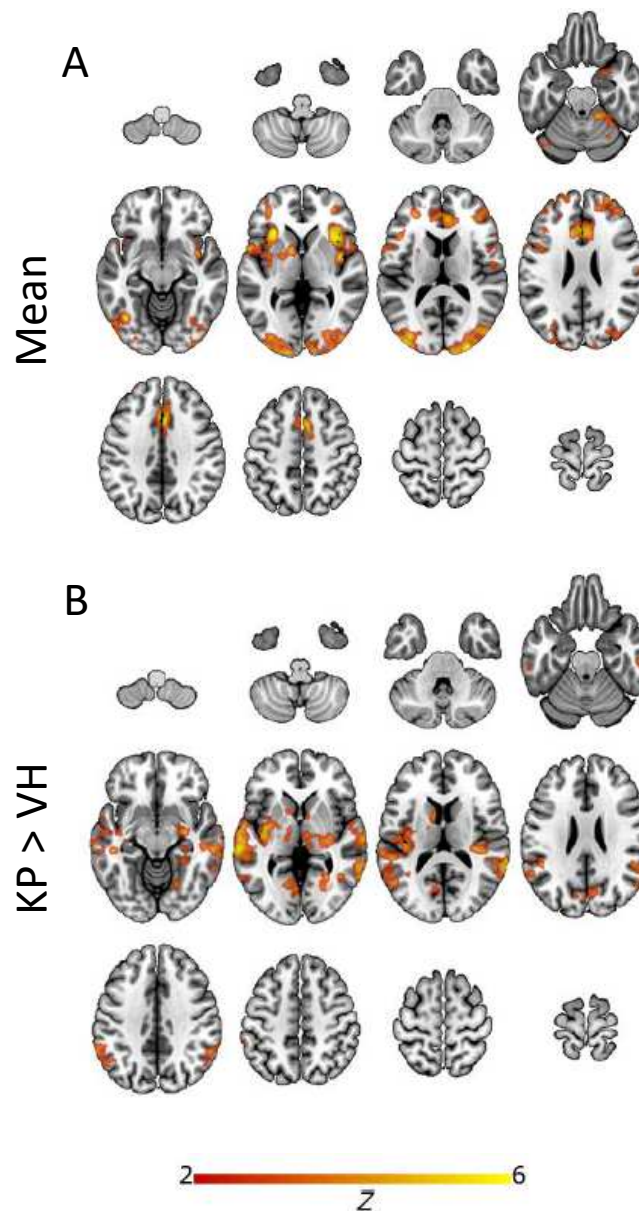
2.5.4 Olfactory Task

Whole brain voxel-wise analysis

Group-mean analysis of the scent trials against baseline for all participants and both study visits showed increased activity in key components of the limbic and olfactory pathways including the amygdala and insula which confirmed that the task and olfactory stimulus worked effectively (Figure 2.7A).

Subsequently, a treatment comparison analysis was carried out on a whole brain voxel-wise level to assess differences in brain response between kisspeptin and vehicle visits (Figure 2.7B). This showed that kisspeptin significantly enhanced brain activity compared to vehicle in key limbic areas related to olfaction and sexual processing in response to a pleasant feminine scent. These areas were the amygdala, putamen, globus pallidus, caudate, thalamus, para-hippocampal gyrus and temporal regions.

Figure 2.7: Olfactory task and treatment effects on whole brain analysis



A: Group mean analysis results for the main effects of the scent trials in all participants and both treatments (kisspeptin and vehicle) relative to baseline (N = 33).

B: Within-subject whole-brain analysis of the effects of kisspeptin (KP) vs. vehicle (VH) for the scent stimulus (N = 33).

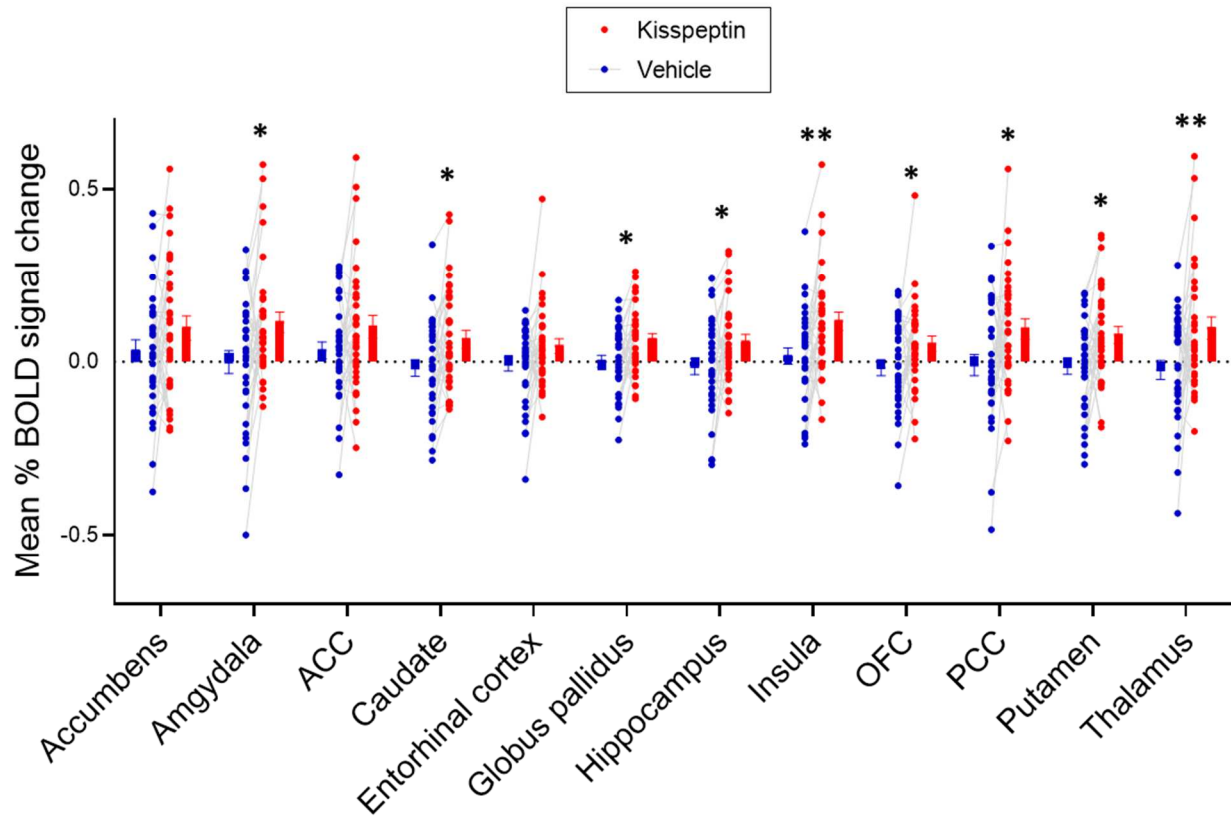
Positive voxel values (red-yellow) represent an increase in activity during kisspeptin treatment compared to vehicle. All statistical maps thresholded at $z = 2.3$, $p < 0.05$ (cluster corrected for multiple comparisons).

Regions of interest analysis

Paired two-tailed t-tests were performed for the set of *a priori* defined anatomical regions which confirmed the findings of the whole brain analysis. Kisspeptin significantly enhanced BOLD activation across 9 out of 12 pre-defined ROIs (Figure 2.8). This significant effect was present in the amygdala: $t(32) = 2.743$, $p = 0.01$; caudate: $t(32) = 2.615$, $p = 0.013$; globus pallidus: $t(32) = 2.566$, $p = 0.015$; hippocampus: $t(32) = 2.235$, $p = 0.033$; insula: $t(32) = 3.105$, $p = 0.004$; orbitofrontal cortex: $t(32) = 2.405$, $p = 0.022$; PCC: $t(32) = 2.303$, $p = 0.028$; putamen: $t(32) = 2.702$, $p = 0.011$; and thalamus: $t(32) = 2.787$, $p = 0.009$.

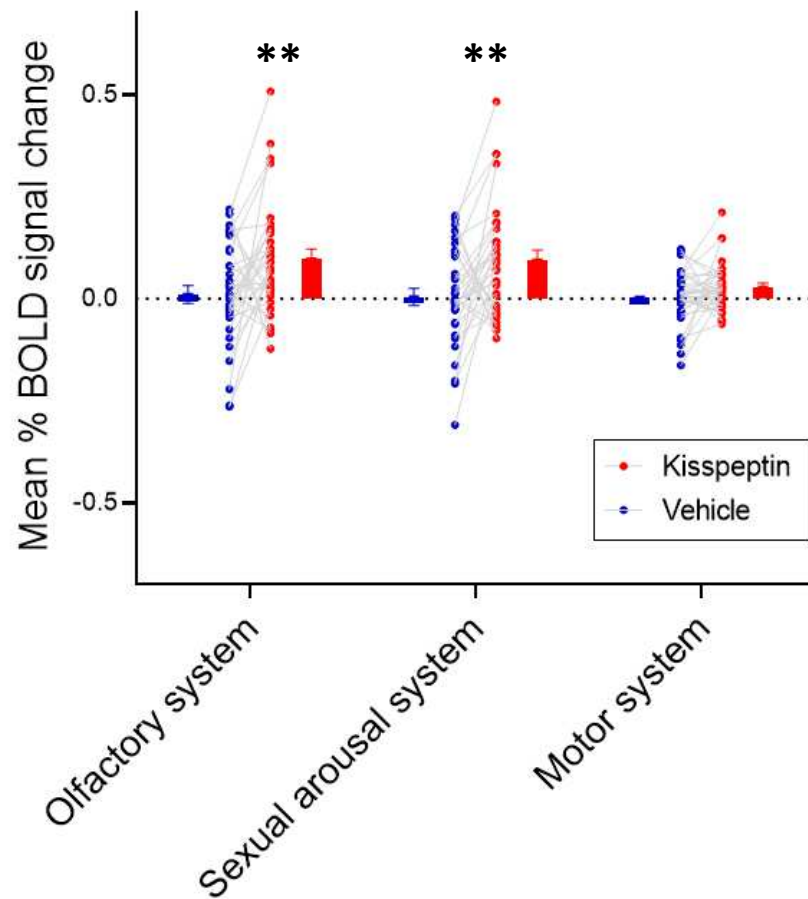
A secondary ROI analysis was performed using brain masks derived from meta-analytic data within the Neurosynth database (Yarkoni *et al.*, 2011) to assess the overall kisspeptin effect compared to vehicle on two further ROIs; the olfactory system and sexual arousal system, with the motor system as a control. The results showed that kisspeptin significantly enhanced brain activity in both the olfactory and sexual arousal systems but not in the control motor system (Figure 2.9). Collectively, these data indicate that kisspeptin augments olfactory as well as sexual and emotional processing in a specific manner, in response to a pleasant feminine olfactory stimulus.

Figure 2.8: ROI analysis of kisspeptin-enhanced activity during the olfactory task



Kisspeptin enhancement (compared to vehicle) of mean %BOLD signal change in 12 *a priori* anatomically defined ROIs (amygdala: $t(32) = 2.743$, $p = 0.01$; caudate: $t(32) = 2.615$, $p = 0.013$; globus pallidus: $t(32) = 2.566$, $p = 0.015$; hippocampus: $t(32) = 2.235$, $p = 0.033$; insula: $t(32) = 3.105$, $p = 0.004$; orbitofrontal cortex: $t(32) = 2.405$, $p = 0.022$; PCC: $t(32) = 2.303$, $p = 0.028$; putamen: $t(32) = 2.702$, $p = 0.011$; thalamus: $t(32) = 2.787$, $p = 0.009$). Data depict within participant paired raw data, mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, paired two-tailed t-tests ($N = 33$).

Figure 2.9: Systems-based ROI analysis of kisspeptin-enhanced activity during the olfactory task



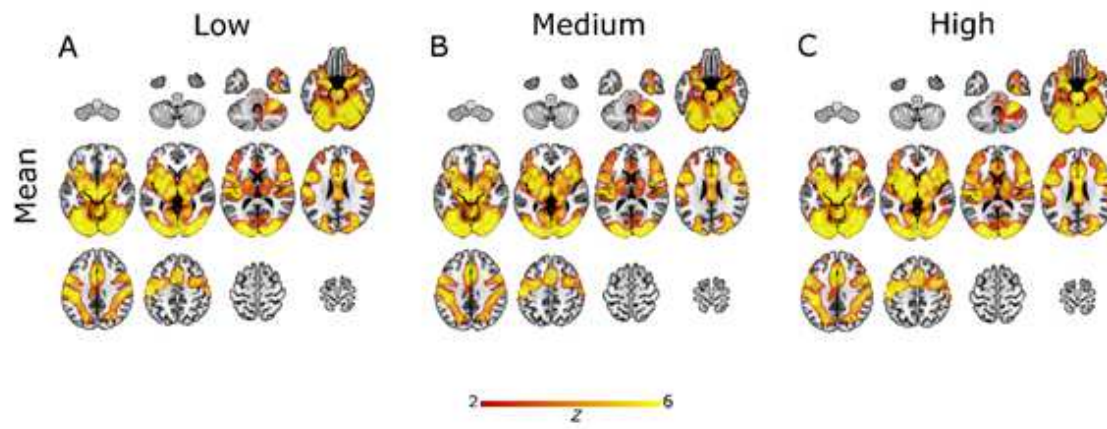
Kisspeptin enhancement (compared to vehicle) of mean %BOLD signal change in three functionally defined ROI brain masks during olfactory task; olfactory system ($t(32) = 2.81$, $p = 0.008$), sexual arousal system ($t(32) = 2.937$, $p = 0.006$), motor system ($t(32) = 1.601$, $p = 0.119$). Data in graphs depict within participant paired raw data, mean $\pm$ SEM. ** $p < 0.01$, paired two-tailed t-tests ($N = 33$).

2.5.5 Facial Attraction Task

Whole brain voxel-wise analysis

Group-mean analysis was performed for each image category (low, medium and high attractiveness) across all participants and both visits. This showed strong BOLD activation in the frontal areas, visual cortex and striatum, confirming that the task worked effectively (Figure 2.10). Next, a treatment comparison analysis was carried out on a whole brain voxel-wise level to assess differences in brain response between kisspeptin and vehicle visits (Figure 2.11). This showed that participants viewing faces rated high and medium attractiveness exhibited enhanced activity in the mPFC and the SFG during kisspeptin administration compared to vehicle. Participants viewing faces rated low attractiveness showed kisspeptin enhanced activity in the SFG but not in the mPFC.

Figure 2.10: Task effects for the facial attractiveness task

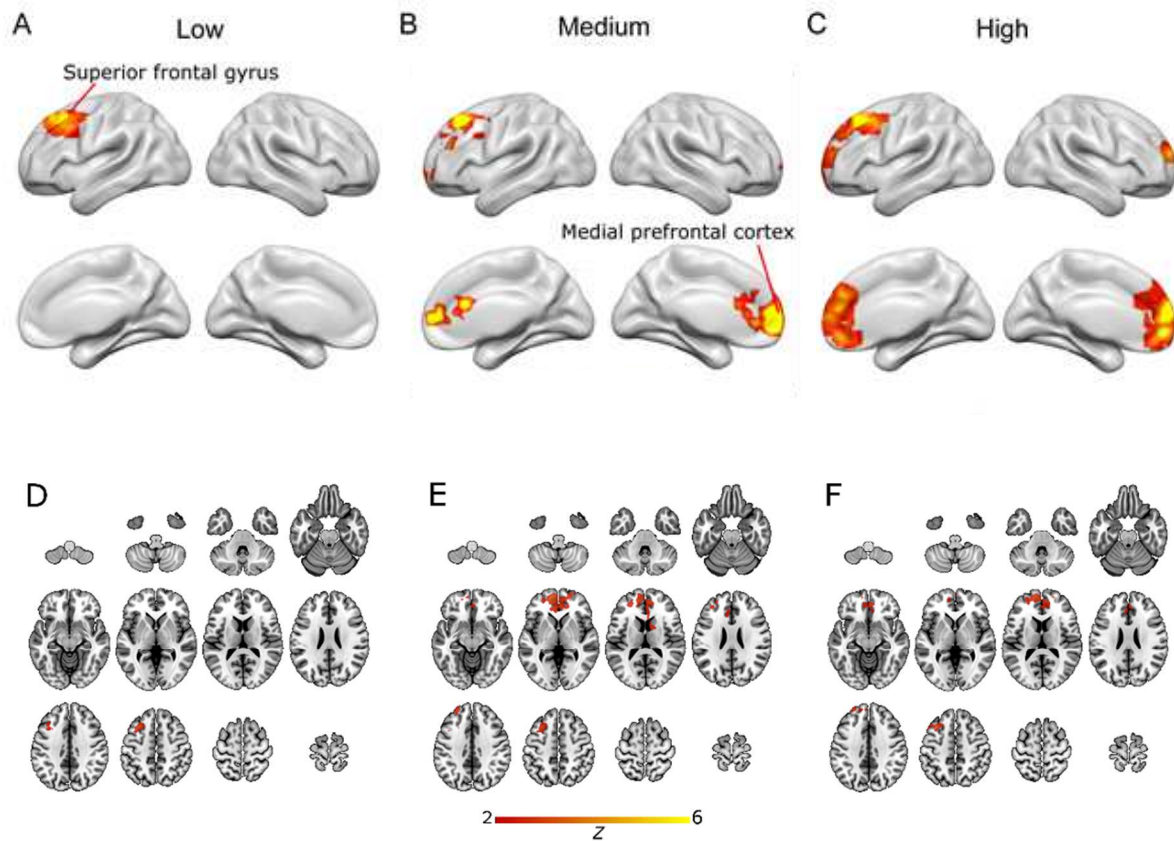


Analyses of the main effects of stimulus type (all subjects, both treatments averaged) for faces rated low **(A)**, medium **(B)** and high **(C)** attractiveness (N = 33).

Positive voxel values represent an increase in activity during images compared to inter-trial intervals.

All statistical maps thresholded at $z = 2.3$, $p < 0.05$ (cluster corrected for multiple comparisons).

Figure 2.11: Whole-brain analysis of kisspeptin-enhanced BOLD activity during the facial attractiveness task



Whole-brain analysis of kisspeptin enhanced BOLD activity compared to vehicle in response to faces rated low, medium and high attractiveness (N = 33).

(A-C) Sagittal views showing enhancement of superior frontal gyrus and medial pre-frontal cortex activity by kisspeptin. **(D-F)** Cross-sectional slices showing the same findings as **(A-C)** respectively.

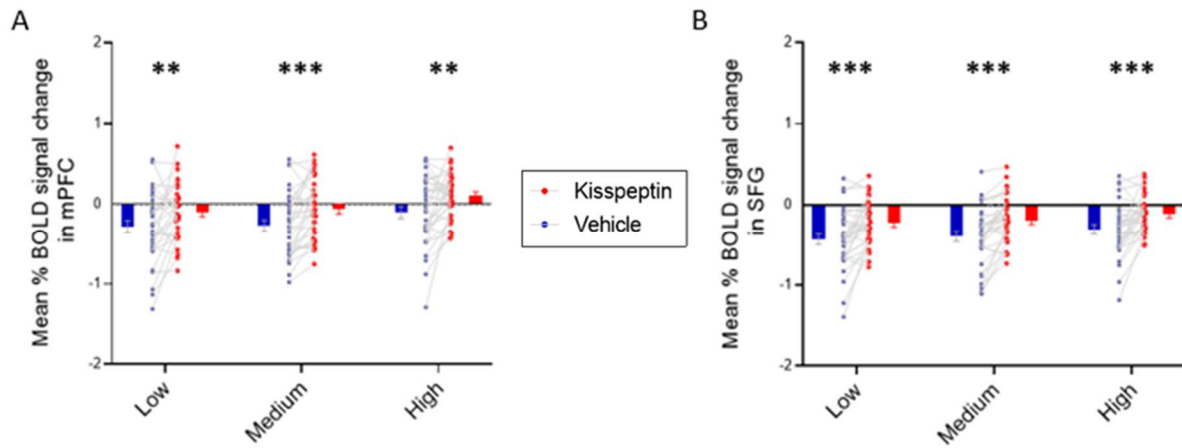
Positive voxel values represent increased activity during kisspeptin infusion compared to vehicle.

Regions of interest analysis

As discussed in the chapter introduction, the mPFC is a well-established area involved in the appreciation of facial beauty (Aharon *et al.*, 2001; O'Doherty *et al.*, 2003). The SFG has also been identified on functional neuroimaging as a responsive area to facial beauty (Winston *et al.*, 2007). Additionally both the mPFC and SFG are known to express kisspeptin receptors in humans (Muir *et al.*, 2001; Kotani *et al.*, 2001). I therefore functionally defined the mPFC and SFG as ROIs based on the group mean results and undertook ROI analyses of both regions. In keeping with their roles as aesthetic brain areas (Aharon *et al.*, 2001; Winston *et al.*, 2007), graded responses in overall mPFC and SFG activity were observed across categories of increasing facial attractiveness in both kisspeptin and vehicle groups (Figure 2.12). Furthermore, kisspeptin significantly enhanced brain activity compared to vehicle in the mPFC and SFG across all categories of attractiveness (Figure 2.12) (mPFC: low: $t(32) = 2.804$, $p = 0.009$; medium: $t(32) = 4$, $p = 0.0004$; high: $t(32) = 3.066$, $p = 0.004$; SFG: low: $t(32) = 3.966$, $p = 0.0004$; medium: $t(32) = 4.567$, $p < 0.0001$; high: $t(32) = 3.668$, $p = 0.0009$). Together, these findings demonstrate that kisspeptin augments the processing of facial beauty across a spectrum of facial attractiveness and may therefore serve as an amplifier within the human neural aesthetic circuitry involved in the assessment of facial beauty.

Additionally, ROI analyses were performed using *a priori* defined limbic brain regions that are known to express kisspeptin receptors and limbic regions involved in sexual arousal (accumbens, amygdala, ACC, caudate, globus pallidus, hippocampus, PCC, putamen and thalamus) (Muir *et al.*, 2001; Comninou *et al.*, 2017). The results did not show any significant difference in brain activity between kisspeptin and vehicle visits in these regions, thus confirming that kisspeptin's enhancement of brain activity on viewing a female face was specific to the established aesthetic regions of the mPFC and SFG (Figure 2.13).

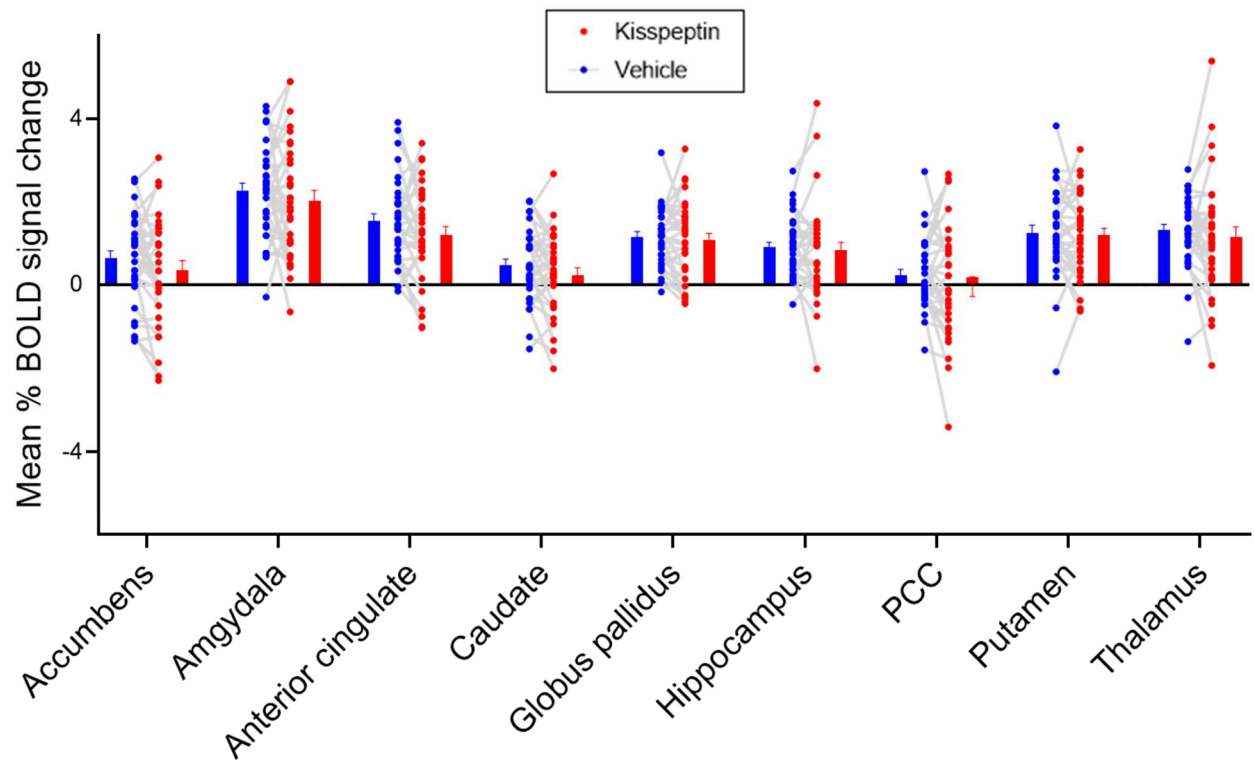
Figure 2.12: ROI analysis of kisspeptin-enhanced activity in mPFC and SFG



(A) Mean %BOLD signal change in the functionally defined ROI: medial prefrontal cortex (mPFC), low: $t(32) = 2.804$, $p = 0.009$; medium: $t(32) = 4$, $p = 0.0004$; high: $t(32) = 3.066$, $p = 0.004$. ** $p < 0.01$, *** $p < 0.001$, paired two-tailed t-tests (N=33).

(B) Mean %BOLD signal change in the functionally defined ROI: superior frontal gyrus (SFG), low: $t(32) = 3.966$, $p = 0.0004$; medium: $t(32) = 4.567$, $p < 0.0001$; high: $t(32) = 3.668$, $p = 0.0009$. ** $p < 0.01$, *** $p < 0.001$, paired two-tailed t-tests (N=33).

Figure 2.13: ROI analyses of kisspeptin-enhanced activity in *a priori* anatomically defined regions, during the facial attractiveness task



ROI analysis based on *a priori* defined brain regions comprising areas known to express kisspeptin receptors and areas involved in sexual arousal, which shows no significant effect of kisspeptin across all face conditions. Data in graph depict within participant raw data, mean $\pm$ SEM (N = 33).

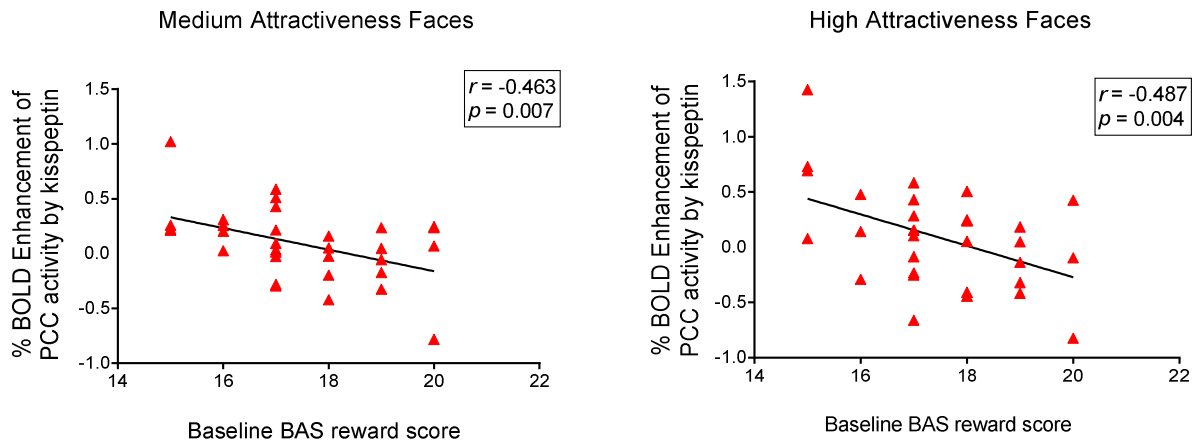
2.5.6 Task ratings

Participants were also asked to rate the olfactory and visual stimuli during the fMRI tasks. While all participants found the scent to be pleasant and feminine, paired *t*-tests did not show any differences in ratings between kisspeptin and vehicle visits. Participants' ratings of the faces were in accordance with ratings given by independent raters (Ma *et al.*, 2015) but paired *t*-tests also revealed no differences in attractiveness ratings between kisspeptin and vehicle visits.

2.5.7 Correlation analyses between kisspeptin-enhanced brain activity and psychometric measures

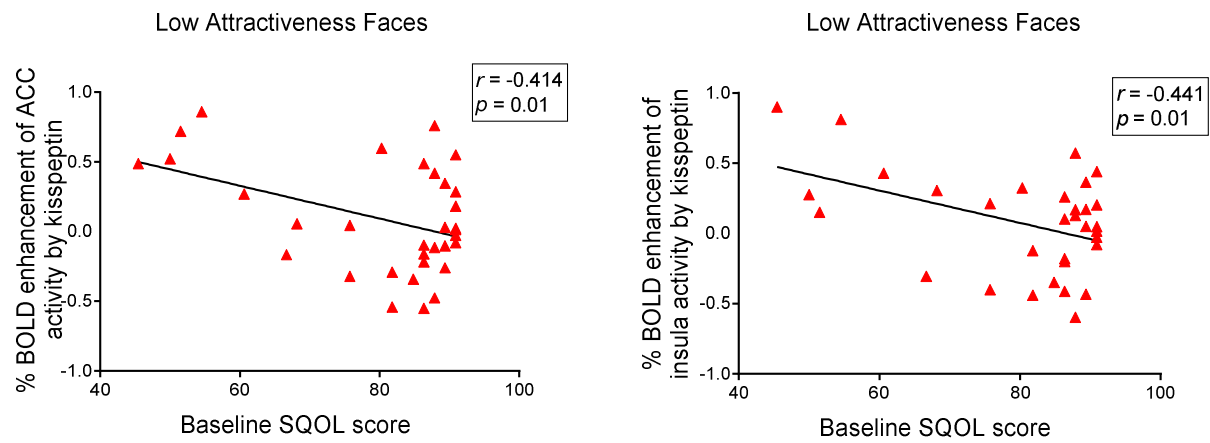
To assess behavioural and functional relevance of the brain activity data, correlation analyses between psychometric parameters and neuroimaging data were carried out. The results demonstrated that participants with lower baseline behavioural activation system (BAS) reward scores (Carver C.S, 1994) showed greater kisspeptin enhanced brain activity in the PCC, on viewing faces rated high ($r = -0.487$, $p = 0.004$, Figure 2.14) and medium attractiveness ($r = -0.463$, $p = 0.007$, Figure 2.14). Similarly, participants who reported a lower baseline sexual quality of life (SQOL) score (Abraham *et al.*, 2008) showed greater kisspeptin-enhanced activity in the ACC ($r = -0.414$, $p = 0.01$) and the insula ($r = -0.441$, $p = 0.01$) on viewing faces rated low attractiveness (Figure 2.15). These behavioural findings provide some functional relevance for the brain activity data and suggest potential future clinical applications of kisspeptin.

Figure 2.14: Correlation between baseline BAS reward score and kisspeptin-enhanced activity in the PCC



Pearson correlation analysis of BAS reward score and PCC enhancement by kisspeptin in response to faces rated medium ($r = -0.463$, $p = 0.007$) and high ($r = -0.487$, $p = 0.004$) attractiveness ($N = 33$).

Figure 2.15: Correlation between baseline SQOL score and kisspeptin-enhanced activity in the ACC and insula



Pearson correlation analysis of baseline SQOL score and kisspeptin-enhancement of ACC ($r = -0.414$, $p = 0.01$) and insula ($r = -0.441$, $p = 0.01$) activity in response to faces rated low attractiveness ($N = 33$).

2.6 Discussion

The findings of this study demonstrate for the first time that the reproductive hormone kisspeptin enhances brain activity in response to olfactory and visual cues of attraction in healthy men.

During the olfactory task, kisspeptin significantly increased brain responses to a pleasant feminine scent in several key components of the limbic system, which are involved in olfactory processing, hedonic valuation of olfactory stimuli and sexual arousal (Fjaeldstad *et al.*, 2017; Ferretti *et al.*, 2005). These included the amygdala, a central component of the primary olfactory cortex, as well as the hippocampus, insula and OFC, which form key limbic projections from the primary olfactory cortex and are common substrates for olfactory and emotional processing (Fjaeldstad *et al.*, 2017; Ferretti *et al.*, 2005). Furthermore, kisspeptin significantly enhanced activity in the globus pallidus and putamen, which together with the amygdala, hippocampus, and OFC constitute the olfactory hedonic processing network (Zou *et al.*, 2016). Interestingly, regions associated with reward, motivation and romantic love were also enhanced by kisspeptin, including the thalamus, PCC and caudate (Acevedo *et al.*, 2012; Aron *et al.*, 2005). Moreover, kisspeptin's enhancement of thalamus and insula activity correspond to established areas of activation during physiological sexual arousal (Kühn & Gallinat, 2011). Importantly, kisspeptin did not affect the motor system which was used as a control (Figure 2.9), thus highlighting the specificity of its effects on olfactory and sexual processing.

The same enhancement in limbic activity was not seen during the facial attractiveness task (Figure 2.13). Instead kisspeptin significantly enhanced activity in the mPFC and SFG, two prefrontal regions that are known to govern the perception of facial beauty (O'Doherty *et al.*, 2003; Ishai, 2007; Winston *et al.*, 2007). This is in keeping with previous data indicating that limbic reward pathways are not necessarily involved in facial aesthetic assessment (Aharon *et al.*, 2001). Thus, these data demonstrate that kisspeptin's effects on brain activity are

specific to the relevant regions involved in different sensory modalities of attraction (olfactory or visual).

In this study peripheral kisspeptin administration was able to enhance brain regions matching areas where human kisspeptin receptor is expressed (Muir *et al.*, 2001; Kotani *et al.*, 2001), suggesting a direct receptor-mediated action of kisspeptin in these brain regions. These findings lead to the question of how peripherally administered kisspeptin can reach brain regions of interest. Gonadotrophin-releasing hormone (GnRH) neurons extend dendritic terminals beyond the blood brain barrier, thus peripheral kisspeptin is capable of stimulating GnRH and in turn, LH without needing to cross the blood brain barrier (Messenger *et al.*, 2005). However, different isoforms of kisspeptin have been shown to have varying degrees of blood brain barrier penetrance (d'Anglemont de Tassigny *et al.*, 2017). Peripherally administered kisspeptin-54 as used in this study, is capable of reaching both GnRH cell bodies beyond the blood brain barrier (d'Anglemont de Tassigny *et al.*, 2017) as well as numerous limbic brain structures known to express kisspeptin and its receptor (Comninou *et al.*, 2017).

It is also important to consider additional pathways that may be involved in exerting the observed downstream effects of kisspeptin on the brain. Kisspeptin activates GnRH neurons and these have been identified in several areas of the brain in humans including the cerebellum, thalamus, anterior olfactory areas, amygdala, stria terminalis, ventral pallidum and putamen (Kubek *et al.*, 1979; Rance *et al.*, 1994; Stopa *et al.*, 1991). However, kisspeptin-enhanced brain activity was also seen in several areas that are not known to contain GnRH neurons or GnRH receptors in humans (including the caudate, globus pallidus, insula and PCC) (Kubek *et al.*, 1979; Rance *et al.*, 1994; Stopa *et al.*, 1991; Wilson *et al.*, 2006), which suggests there are GnRH-independent actions of kisspeptin in these regions. Furthermore, recent functional evidence indicates that certain kisspeptin-induced sexual behaviours can occur independently of GnRH. These include kisspeptin-induced lumbar lordosis in mice (Hellier *et al.*, 2018) and kisspeptin-induced erections in rats (Gresham *et al.*, 2016). Data from

animal studies demonstrate that kisspeptin also interacts with numerous other neuropeptide systems including serotonin (Nathan *et al.*, 2015a; Tanaka *et al.*, 2013), dopamine (Pineda *et al.*, 2017), vasopressin (Pineda *et al.*, 2017), gamma-aminobutyric acid (Di Giorgio *et al.*, 2014), glutamate (Nathan *et al.*, 2015b) and nitric oxide (Hanchate *et al.*, 2012; Hellier *et al.*, 2018; Tena-Sempere, 2017). Thus, the fMRI changes that are seen here may comprise direct kisspeptin effects on its receptor as well as interactions between kisspeptin and other downstream neural systems.

Furthermore, significant correlations were observed between kisspeptin-enhanced brain activity and important psychometric parameters thereby providing behavioural and functional relevance for the observed brain changes. Greater kisspeptin enhancement was observed in the PCC on viewing attractive faces in men with lower baseline reward scores. The PCC is implicated in romantic love (Aron *et al.*, 2005) and its activity is known to vary with emotional memory and reward (Pearson *et al.*, 2011). Thus, kisspeptin may act to enhance emotional salience and reward processing in the PCC on viewing attractive faces in order to re-balance a lower reward drive, in favour of promoting sexual attraction. A similar relationship was demonstrated between kisspeptin's enhancement of ACC and insula activity in response to less attractive female faces and low sexual quality of life. The ACC is a dopamine-rich area which responds to visual sexual stimuli (Oei *et al.*, 2012) and both the ACC and insula are areas implicated in sexual arousal (Arnow *et al.*, 2001), facial attraction (O'Doherty *et al.*, 2003; Winston *et al.*, 2007) and motivation towards reward (Holroyd & Yeung, 2012; Murty *et al.*, 2016). Therefore, kisspeptin's enhancement of these brain regions on viewing less attractive faces may serve to strengthen feelings of reward, attraction and incentive motivation in individuals experiencing lower sexual quality of life. Collectively these findings provide key behavioural and functional relevance for kisspeptin's enhancement of brain activity on viewing female faces and lay the foundations for potential clinical applications for kisspeptin in patients with psychosexual disorders.

2.6.1 Limitations

There are some limitations to this study. Although circulating kisspeptin levels achieved in this study are similar to the levels observed in normal physiological pregnancy (Horikoshi *et al.*, 2003) and are equivalent to the levels required to restore reproductive function in women with hypothalamic amenorrhoea (Jayasena *et al.*, 2014b), healthy men have low circulating kisspeptin levels (Horikoshi *et al.*, 2003). Thus, exogenous administration of peripheral kisspeptin in healthy men may have differing effects on brain processing compared to endogenous effects of circulating kisspeptin in the brain. Encouragingly, there were no differences in circulating hormones including testosterone and cortisol between kisspeptin and vehicle infusions thus negating these potential confounders (Figure 2.7).

Another potential limitation of this study is the use of a manufactured feminine scent rather than a naturally occurring opposite-sex olfactory stimulus. In animal studies, opposite-sex odours or pheromones have been widely used to elicit olfactory-driven behavioural and endocrine responses (Petrulis, 2013; Watanabe *et al.*, 2017; Aggarwal *et al.*, 2018; Hellier *et al.*, 2018). However, in humans the evidence for behavioural and physiological responses to opposite-sex odours is conflicting and during the planning of the methodology, a reliable and validated natural female pheromone was not readily available (Wyatt, 2015). Therefore Chanel No.5 was selected as a well-recognised and validated feminine scent known to activate brain areas responding to a consciously perceived feminine olfactory cue (Huh *et al.*, 2008) so that kisspeptin's effects on these regions could be robustly assessed. Although all participants in the study rated this scent as both pleasant and feminine at baseline, no differences in scent ratings were demonstrated between kisspeptin and vehicle visits. Reasons for this may be that a 20-point Likert scale was used to assess scent ratings. A 100-point visual analog scale may have been more sensitive to detect smaller changes in olfactory perception (Sung & Wu, 2018). Furthermore, the fMRI environment is somewhat artificial and scent ratings were obtained at the end of the scan session at which stage, fatigue and boredom may have affected ratings. Similarly, although participants rated the female faces very closely to the

scores given by independent raters, there were also no observed differences between kisspeptin and vehicle visits. In this task, due to limitations of the equipment, a five-point Likert scale was used to assess level of attractiveness, which may also have been too insensitive to detect subtle changes (Sung & Wu, 2018). Nevertheless, it is important to note that the primary aim of this study was to detect changes in brain responses as measured by BOLD activity during kisspeptin infusion and therefore the study was not powered to detect behavioural changes such as attractiveness and olfactory ratings.

2.6.2 Summary

In summary, the results of this study reveal a previously undescribed kisspeptin-activated attraction pathway, uniting two major sensory inputs of human attraction with corresponding neural processing and reproductive hormonal control. Furthermore, kisspeptin's enhancement of key brain areas within these pathways is augmented in men with lower behavioural reward drive and sexual quality of life providing functional relevance for the imaging findings. Collectively, this has important implications for our understanding of human psychosexual function and its endocrine control. As such, this introduces a new and exciting avenue for manipulation of these kisspeptin-mediated pathways to deliver clinical strategies for individuals suffering from related reproductive and psychosexual disorders.

2.6.3 Future work

As discussed previously, this study used exogenous kisspeptin to elicit changes in brain activity in response to olfactory and visual cues of attraction, thus unravelling a new and exciting therapeutic potential for the reproductive hormone kisspeptin. However, further studies are necessary to fully elucidate the role of endogenous kisspeptin activity in human psychosexual function.

This fMRI paradigm is also a useful tool for investigating the effects of kisspeptin on brain responses to other sensory modalities such as audition (Asaba *et al.*, 2017). A novel role for

kisspeptin has been demonstrated in human olfactory processing and while the subject is contentious, further work studying the effects of kisspeptin on brain responses to opposite-sex odours using stimuli such as clothing worn by different women or women in various phases of the menstrual cycle (Gildersleeve *et al.*, 2012; Miller & Maner, 2010) would be highly interesting. Additionally, fMRI tasks involving non-sexual stimuli such as food odours or food images would also be relevant given the role of kisspeptin in food intake and metabolism seen in animal studies (Greives *et al.*, 2011; Navarro & Tena-Sempere, 2012).

When considering attraction, it is also important to note that although visual and olfactory inputs are fundamental cues of attraction in many species (Fisher, 1998), human sexual responses are complex and influenced by multiple additional components such as context, body language and personality traits (Moore, 2010) which may impact individual perceptions of attractiveness. The study protocol controlled for some of these factors by omitting motion picture and audio stimuli as potential confounders, however it would be interesting to study these other components of attraction in future.

Chapter 3

The effects of peripheral kisspeptin administration on appetite and food intake in healthy men.

3.1 Introduction

Reproduction and metabolism are intricately linked and are essential for survival. In humans, a minimum body weight is required to trigger pubertal development (Frisch & Revelle, 1970) and both weight loss and excessive weight gain can lead to hypogonadism in adults (Vigersky *et al.*, 1977; Vermeulen *et al.*, 1993). Similarly, patients with acquired or congenital hypogonadism exhibit increased adiposity, namely visceral adiposity, and reduced muscle mass compared to healthy controls (Carrageta *et al.*, 2019). Testosterone replacement has been shown to improve body composition with reduced fat mass and increased lean mass in hypogonadal men (Isidori *et al.*, 2005; Corona *et al.*, 2014). However, when compared to age-matched eugonadal controls, testosterone is not able to fully ameliorate changes in adiposity and metabolic derangements in hypogonadal men (Svartberg *et al.*, 2008), which points to additional factors involved in the interplay between the reproductive axis and metabolic pathways that remain unexplored.

Furthering our knowledge of the links between metabolism and reproduction has major clinical significance, as obesity is a critical public health issue affecting all age groups and populations worldwide, with current estimates that two thirds of adults in high income countries are overweight or obese (Ritchie & Roser, 2020). In addition to the adverse cardiometabolic outcomes associated with obesity, there is also a significant detrimental impact on reproduction and fertility (Ramlau-Hansen *et al.*, 2007; Palmer *et al.*, 2012; Broughton & Moley, 2017).

Kisspeptin is a hypothalamic neuropeptide with a well-characterised role in reproduction and fertility (Seminara *et al.*, 2003; Dhillon *et al.*, 2005; Jayasena *et al.*, 2014a). As discussed in Chapter 1, there is also increasing evidence that kisspeptin is involved in central brain pathways controlling appetite and feeding behaviours. Kisspeptin neurons form neuroanatomical connections with hypothalamic feeding centres including AgRP/NPY and POMC/CART neurons (Backholer *et al.*, 2010; Manfredi-Lozano *et al.*, 2016; Nestor *et al.*,

2016). However, the literature surrounding the functional relationships between kisspeptin and these appetite-regulating neuropeptides is limited. *In vitro* studies demonstrate that kisspeptin inhibits NPY neuronal activation and stimulates POMC neuronal activation (Fu & Van Den Pol, 2010), whereas *in vivo* studies in sheep show that kisspeptin increases NPY expression and decreases POMC expression (Backholer *et al.*, 2010) which conflicts with findings in desert jerboa and dwarf hamsters in which kisspeptin increases POMC expression (Talbi *et al.*, 2016; Cázares-Márquez *et al.*, 2019). Leptin is another appetite-regulating peptide with a permissive role in mediating the onset of puberty under adequate nutritional conditions (Farooqi & O'Rahilly, 2009). Kiss1 neurons are known to co-express the leptin receptor (Donato Jr *et al.*, 2011), however direct kisspeptin-leptin interactions do not appear to be essential in mediating reproductive responses to metabolic signals (Cravo *et al.*, 2013). Taken together, this suggests that kisspeptin signalling has a role in metabolic pathways, however the neuro-humoral components that interface with kisspeptin within reproductive and metabolic networks have yet to be fully uncovered.

Both central and peripheral kisspeptin administration can acutely suppress feeding behaviour in a dose-dependent manner in mice (Stengel *et al.*, 2011; Dong *et al.*, 2019). However acute and chronic central or peripheral kisspeptin administration does not affect food intake in male rats (Thompson *et al.*, 2004; Castellano *et al.*, 2005; Thompson *et al.*, 2006), which may reflect differences in experimental conditions and species/strain chosen.

Taken together, it is established that kisspeptin is sensitive to internal and external signals regulating appetite and energy balance and it is a key integrator between nutritional cues and the reproductive axis. Although there is currently a limited understanding regarding the interactions between kisspeptin and appetite-regulating pathways, both endogenous kisspeptin-signalling (Tolson *et al.*, 2014; Velasco *et al.*, 2019) and exogenous kisspeptin administration (Stengel *et al.*, 2011; Dong *et al.*, 2019; Saito *et al.*, 2019) have been shown to

alter feeding behaviour in rodents. Thus far, kisspeptin has not been investigated in relation to human appetite and feeding.

3.1.1 Summary

There is a growing body of neuroanatomical and functional evidence pointing to a role for the reproductive neuropeptide kisspeptin within appetite-regulating pathways and food intake. However, until now only limited data exists, and some results are conflicting. This is an exciting and highly relevant area that needs further exploration as obesity is a major public health problem with detrimental effects on fertility and kisspeptin-based therapies are being developed for use in humans to treat reproductive disorders. Thus, it is imperative to characterise the effects of kisspeptin on appetite and food intake in humans. As studies of kisspeptin administration on food intake have been carried out primarily in male rodents, a first-in-human proof-of-concept study involving healthy men would be useful to assess any potential translational findings.

3.2 Aims and hypothesis

Aims: To investigate the effects of peripheral kisspeptin administration compared to vehicle, on appetite and food intake during *ad libitum* meal ingestion in a proof-of-concept study in healthy men.

Hypothesis: Peripheral kisspeptin administration suppresses appetite and food intake in healthy men.

3.3 Methods

3.3.1 Study approval

The study was performed in accordance with the Declaration of Helsinki. All participants gave written informed consent prior to inclusion in the study. The study was approved by the West London Research Ethics Committee (16/LO/0391).

3.3.2 Sample size

A sample size of 15 was chosen in order to give sufficient power to detect a clinically significant reduction in food intake with a hormonal intervention compared to vehicle (placebo). As no similar study of kisspeptin has been performed in humans, this was based on previous clinical studies investigating the effects of other peptide hormones (including glucagon, peptide-YY, glucagon-like peptide-1 and cholecystokinin) which showed a similar sample size was able to detect a reduction in food intake by at least 12% with these peptides (Geary *et al.*, 1992; De Silva *et al.*, 2011; Cegla *et al.*, 2014).

3.3.3 Study recruitment and eligibility criteria

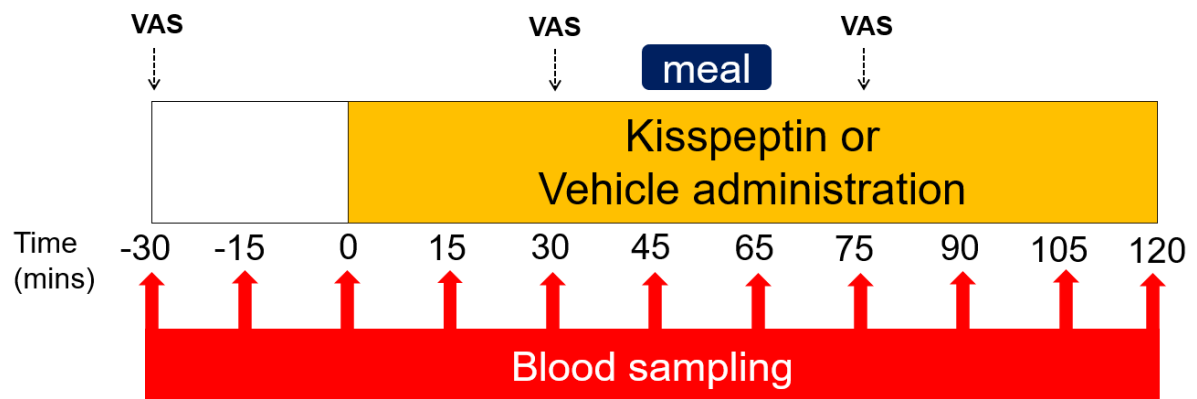
Healthy men were recruited using online and print advertisements. Written informed consent was obtained from each participant prior to study enrolment. Exclusion criteria were applied to avoid confounding factors such as obesity, comorbidities, medications or abnormal eating behaviours (BMI <18.5 kg/m² or >25 kg/m², history of medical and psychological conditions, use of prescription or recreational drugs, treatment with an investigational drug within the preceding two months, blood donation within three months' of study participation, ingestion or inhalation of nicotine-containing substances, alcoholism, and history of cancer). Abnormal eating behaviour was screened out using the Dutch Eating Behaviour Questionnaire (van Strien *et al.*, 1986) and the SCOFF questionnaire (Morgan *et al.*, 1999) (Appendix 4).

3.3.4 Study day protocol

Participants completed two study visits each and received kisspeptin-54 or vehicle (gelofusine) infusions in a randomised, single-blinded crossover protocol (Figure 3.1). Randomisation was performed by an independent investigator (using Research Randomizer, www.randomizer.org). This was a within-participant design study, in which the participants acted as their own control thereby minimising variability and enhancing power. All study visits commenced in the morning to control for circadian hormonal changes. Participants were asked to abstain from strenuous exercise, alcohol, and caffeine for 24 hours prior to each study visit. Each participant was instructed to choose a meal and eat that same meal at 8pm on the night preceding each study visit, fast overnight and attend the morning study visit fasted.

Following acclimatisation and baseline sampling, a kisspeptin-54 infusion (1 nmol/kg/hour) or vehicle (gelofusine at equivalent rate) was commenced at T = 0 minutes and an *ad libitum* meal was presented to participants at T = 45 minutes. The kisspeptin dose was selected based on previous experience establishing it as a bioactive dose that reaches steady state at 30 minutes, prior to *ad libitum* meal ingestion at 45 minutes (Comninou 2017). Kisspeptin or vehicle infusion was stopped at T = 120 minutes after the last blood sample was taken (Figure 3.1). Participants were asked to rate their hunger on 10 cm visual analogue scales at T = -30 minutes (pre-infusion), T = 30 minutes (pre-meal) and T = 75 minutes (post-meal).

Figure 3.1: Study day protocol



Participants attended for two study visits each, one for intravenous administration of kisspeptin-54 (1 nmol/kg/hour) and one for intravenous administration of an equivalent volume of vehicle for 120 minutes. Visual analogue scales (VAS) were completed at -30, 30 and 75 minutes. Blood sampling took place at time-points -30, -15, 0, 15, 30, 45, 65, 75, 90, 105 and 120 minutes to measure circulating kisspeptin, LH and testosterone during the study. The infusion commenced at T = 0 minutes and an *ad libitum* meal was provided at T = 45 minutes, after kisspeptin levels reached steady state.

3.3.5 *Ad libitum* test meal

Studies of *ad libitum* food ingestion are frequently used to assess the potential influence of novel substances and investigational drugs on energy intake (Gregersen *et al.*, 2008; Arvaniti *et al.*, 2000). A single test meal was chosen for this study versus a buffet-style test meal as free access to a buffet meal could lead to variations in the macronutrient composition ingested and affect satiety (Arvaniti *et al.*, 2000). The single *ad libitum* meal study is a simple to administer and reproducible test that closely mimics the physiology of an oral calorie challenge (Gregersen *et al.*, 2008).

Two study meal choices were selected with similar kcal/weight; Waitrose Spaghetti Bolognese (125kcal/100g) or Waitrose Mushroom Risotto (124kcal/100g). In order to determine the choice of study meal, a taste test was performed for each participant prior to their study visits. Participants were asked to rate how much they liked each meal using a Likert scale (Appendix 5), and the meal rated closest to 'neither like nor dislike' was used as the study meal to minimise over- or under-eating due to taste preferences. Participants were instructed to eat until comfortably full. All clocks, electronic devices and reading material were removed and no other people remained in the room while the participants were eating to reduce distractions that might affect the quantity of food consumed (Blass *et al.*, 2006; Hetherington *et al.*, 2006). Energy intake of the *ad libitum* meal was measured in kilocalories.

3.3.6 *Visual analog scales*

Visual analog scales (VAS) are an established psychometric assessment tool in appetite research with good inter-test reliability in healthy adults (Flint *et al.*, 2000; Parker *et al.*, 2004). In this study, 10 cm VAS scales were used in seven questions to assess hunger, satiety, nausea, sleepiness and palatability of the test meal (Appendix 6). VAS scores were recorded at -30 minutes (pre-infusion), 30 minutes (pre-meal), and 75 minutes (post-meal).

3.3.7 Kisspeptin-54 peptide preparation

Human sequence kisspeptin-54 peptide was synthesised by Bachem (St Helens) and aliquoted into glass vials at a final concentration of 600nM/vial. Purification was performed by reverse-phase High Performance Liquid Chromatography. Electrospray mass spectroscopy and amino acid analysis confirmed identity of the peptide. Toxicology testing in animals was conducted prior to administration to human volunteers. The limulus amoebocyte lysate test detected no endotoxin (Associates of Cape Cod, Liverpool, UK), and bacterial culture was sterile (Department of Microbiology, Hammersmith Hospital, London, UK), in samples of kisspeptin-54 peptide. Vials of kisspeptin-54 were freeze-dried and stored at -20°C. Vials were defrosted on the morning of the study and reconstituted in 0.9% saline prior to administration.

3.3.8 Infusions

For the kisspeptin infusions, kisspeptin-54 was dissolved in 1 ml of 0.9% NaCl (Braun) and added to 49 ml of Gelofusine (Braun, Germany). Kisspeptin was infused at a rate of 1 nmol/kg/hour, a dose known to significantly increase LH levels in men (Comninou *et al.*, 2017). Vehicle infusions consisted of Gelofusine (Braun, Germany), administered at the equivalent rate to the kisspeptin infusion for each participant.

3.3.9 Blood sample collection and analyses

Kisspeptin measurement

Blood samples for kisspeptin measurement were collected into lithium heparin tubes containing 5000 kallikrein inhibitor units of aprotinin (200 µl Trasylol, Bayer) to limit enzymatic breakdown of kisspeptin. Immediately after collection, blood samples were centrifuged at 4000 RPM for 4 minutes at room temperature. Plasma was separated and transferred into 1.5 ml microcentrifuge tubes (Eppendorf), stored at -20°C and defrosted on the day of sample analysis.

Measurement of plasma kisspeptin immunoreactivity was performed using an established in-house manual radioimmunoassay (Dhillon *et al.*, 2005) that has been described in detail in Chapter 2 (2.4.14). The lower limit of detection for the assay was 2 pmol/L, the intra-assay coefficient of variation was 8.3% and the inter-assay coefficient of variation was 10.2% (Dhillon *et al.*, 2005).

Measurement of circulating hormones

Blood samples for LH and testosterone measurement were collected into tubes containing clot activator (BD Vacutainer). After collection, blood samples were left to clot at room temperature for ≥ 30 minutes and then centrifuged at 3000 RPM for 10 minutes at room temperature. Serum was separated and transferred into LP4 tubes. All samples were stored immediately at -20°C then defrosted prior to sample analysis.

Circulating LH and testosterone levels were measured using automated chemiluminescent immunoassays (Abbott Diagnostics, Maidenhead, UK). The intra- and inter-assay coefficients of variation were: LH $\leq 7\%$ and testosterone $\leq 8\%$. Analytical sensitivities were: 0.5IU/L (LH) and 2nmol/L (testosterone).

3.3.10 Statistical analyses

Statistical analyses were performed using GraphPad Prism 8 software with statistical significance set at $p < 0.05$. Data were normally distributed by Kolmogorov testing and individual paired two-tailed t-tests were performed to investigate the effects of kisspeptin compared to vehicle on appetite parameters and food intake. Hormone level data analysis was performed using two-way repeated measures ANOVA with mixed effects modelling.

3.4 Results

3.4.1 Participants

Table 3.1 summarises the baseline characteristics of the 15 healthy men who completed this single-blinded, randomised crossover study.

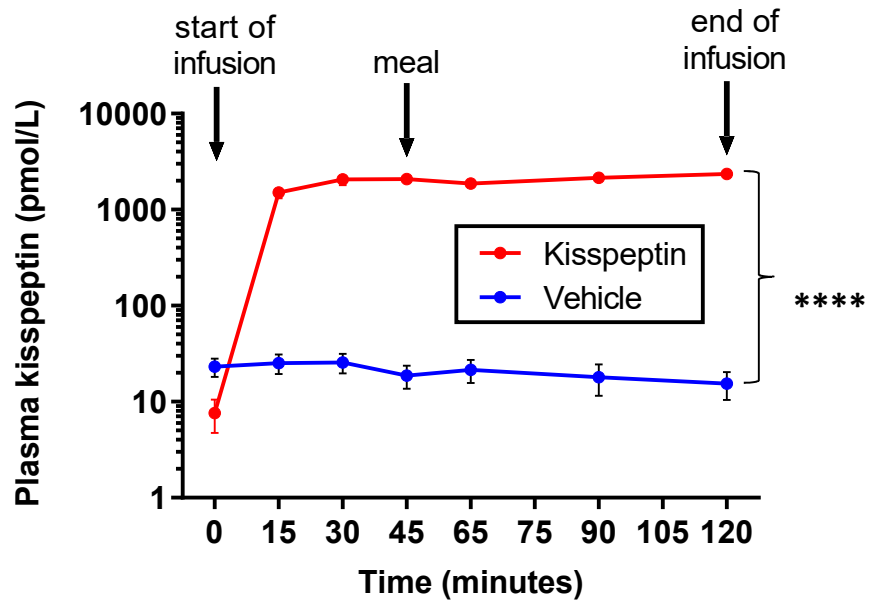
Table 3.1: Baseline characteristics of study participants

	Mean $\pm$ SEM	Range
Age (years)	24.3 $\pm$ 1.00	20 - 32
Weight (kg)	71.23 $\pm$ 2.69	57.2 – 93.7
BMI (kg/m ²)	22.2 $\pm$ 0.56	18.9 – 25.1

3.4.2 Effects of peripheral kisspeptin administration on reproductive hormone levels during the *ad libitum* meal study

In keeping with the findings in Chapter 2 (Figure 2.6) and previously published work (Comninou 2017), peripheral kisspeptin infusion at 1 nmol/kg/hour significantly increased circulating plasma kisspeptin levels, reaching steady state at 30 minutes (Figure 3.2). Consequently, circulating LH levels were also significantly increased during kisspeptin infusion (Figure 3.3A), confirming that the dose of kisspeptin used was biologically active. As expected, kisspeptin infusion had no significant effects on testosterone levels during the 120 minute study period (Figure 3.3B) (Jayasena *et al.*, 2011).

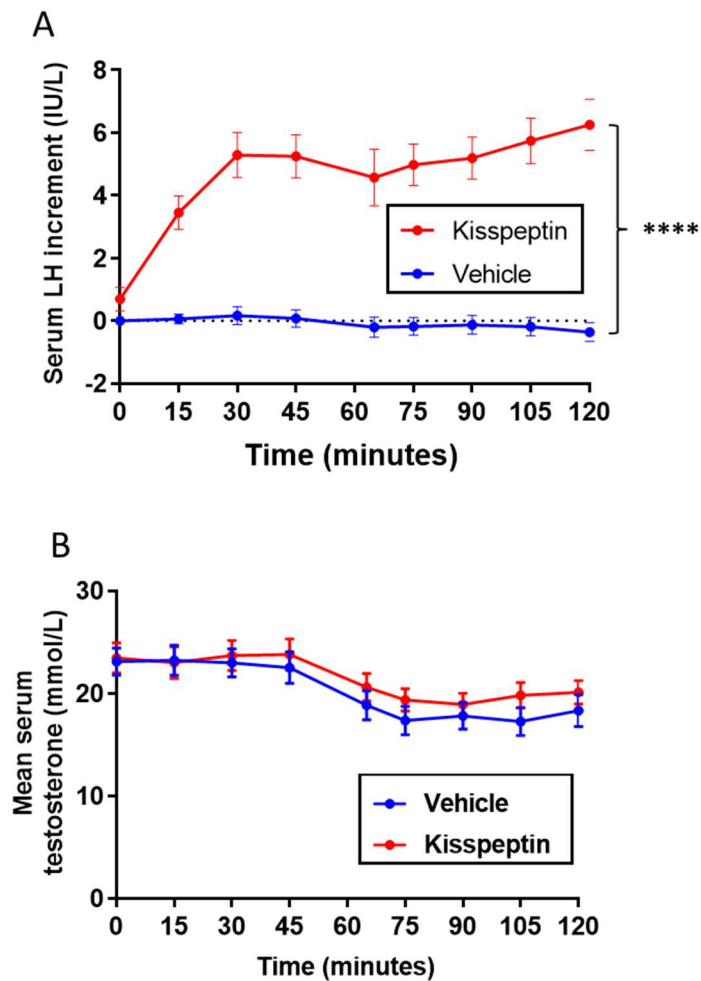
Figure 3.2: Effects of peripheral kisspeptin administration on plasma kisspeptin levels during the *ad libitum* meal study



Plasma kisspeptin levels significantly increased with kisspeptin infusion compared to vehicle and reached steady state for the duration of the *ad libitum* meal. Data depict mean $\pm$ SEM (N = 15).

**** $p < 0.0001$ using two-way repeated measures ANOVA with mixed effects modelling.

Figure 3.3: Effects of peripheral kisspeptin administration on circulating reproductive hormones during the *ad libitum* meal study



(A): Serum luteinising hormone (LH) levels significantly increased with kisspeptin infusion whereas there was no significant difference in testosterone levels (B) between kisspeptin and vehicle infusion. Data depict mean $\pm$ SEM (N = 15). **** $p < 0.0001$ using two-way repeated measures ANOVA with mixed effects modelling.

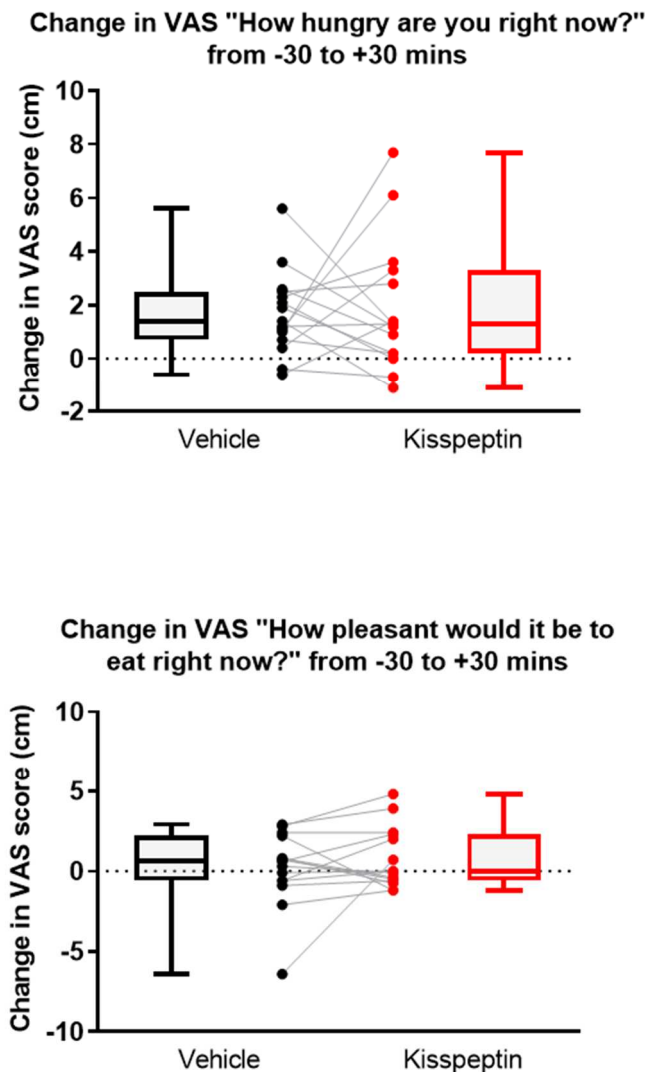
3.4.3 Effects of peripheral kisspeptin administration on appetite

Changes in appetite during the fasted state were assessed using VAS questions; “How hungry are you right now?” and “How pleasant would it be to eat right now?”. Changes in pre-infusion (T = -30 minutes) and pre-meal (T = 30 minutes) scores were not significantly different during kisspeptin visits compared to vehicle visits (Figure 3.4).

Changes in satiety following the *ad libitum* meal were assessed using VAS questions; “How full do you feel right now?” and “How much could you eat right now?”. Similarly, there were no significant differences between pre-meal (T = 30 minutes) and post-meal (T = 75 minutes) scores during kisspeptin and vehicle visits (Figure 3.5).

Reassuringly, participants found the palatability of the *ad libitum* test meal to be comparable during kisspeptin and vehicle visits (Figure 3.6) and there were also no significant differences in other confounding factors i.e. nausea ($t(14) = 0.122$, $p = 0.905$) or sleepiness ($t(14) = 0.560$, $p = 0.585$) as assessed by pre-meal VAS scores between kisspeptin and vehicle visits.

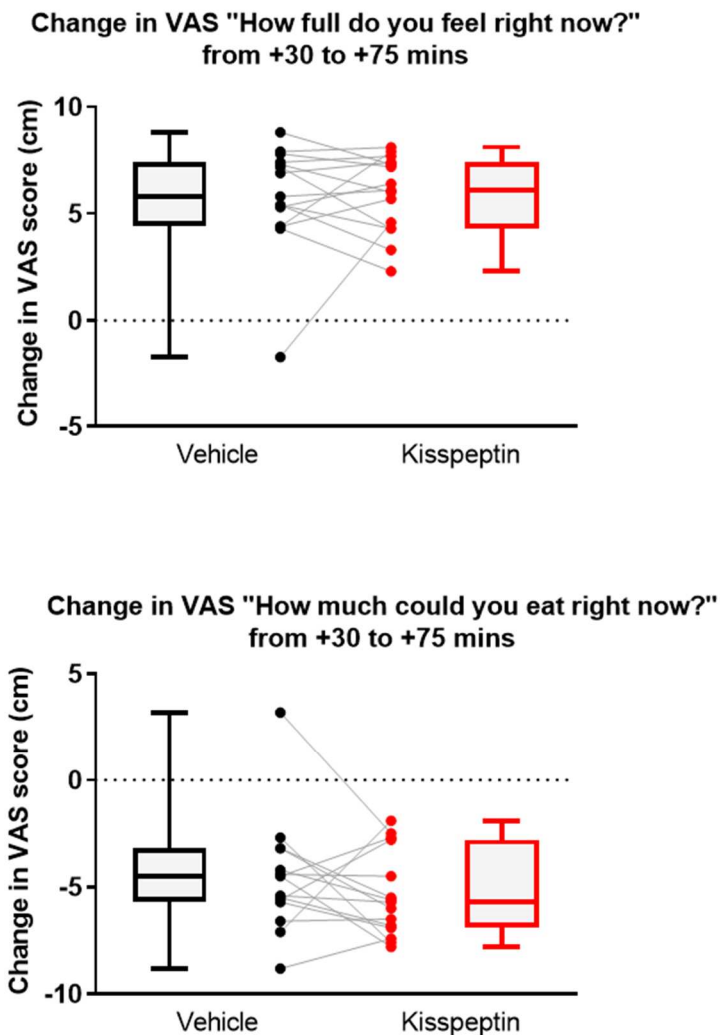
Figure 3.4: Change in pre-meal appetite scores during kisspeptin and vehicle visits



Change in appetite scores from pre-infusion (T = -30 minutes) to pre-meal (T = 30 minutes) during kisspeptin and vehicle infusions. Appetite assessed by VAS questions; "How hungry are you right now?": $t(14) = 0.242$, $p = 0.812$, and "How pleasant would it be to eat right now?": $t(14) = 0.933$, $p = 0.367$, kisspeptin vs vehicle using paired two-tailed t-test (N = 15).

VAS = visual analogue scale. Data presented as within-participant paired raw data and box and whiskers plot depicting maximum, minimum and mean values.

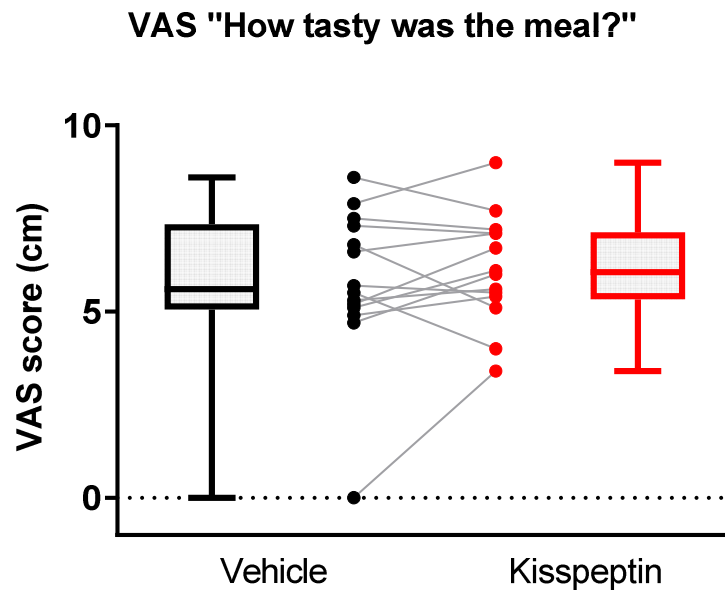
Figure 3.5: Change in fullness scores from pre-meal to post-meal during kisspeptin and vehicle visits



Change in fullness scores from pre-meal (T = 30 minutes) to post-meal (T = 75 minutes) during kisspeptin and vehicle visits. Fullness assessed by VAS questions; "How full do you feel right now?": $t(14) = 0.220$, $p = 0.829$ and "How much could you eat right now?": $t(14) = 1.075$, $p = 0.300$, kisspeptin vs vehicle using paired two-tailed t-tests (N = 15).

VAS = visual analogue scale. Data presented as within-participant paired raw data and box and whiskers plot depicting maximum, minimum and mean values.

Figure 3.6: Palatability of the *ad libitum* meal during kisspeptin and vehicle visits



Palatability of the *ad libitum* test meal assessed at T = 75 minutes by VAS question; "How tasty was the meal?": $t(13) = 0.969$, $p = 0.350$, kisspeptin vs vehicle using paired two-tailed t-tests (N = 15).

VAS = visual analogue scale. Data presented as within-participant paired raw data and box and whiskers plot depicting maximum, minimum and mean values.

3.4.4 Effects of peripheral kisspeptin administration on ad libitum energy intake

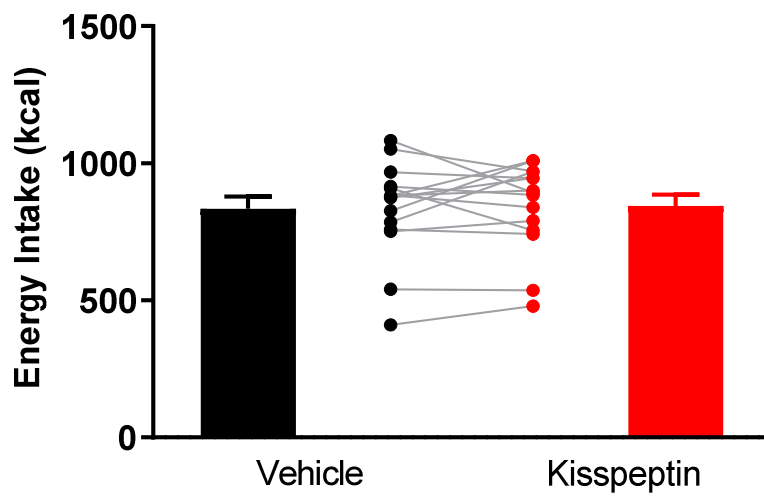
There were no significant differences in *ad libitum* energy intake during kisspeptin infusion compared with vehicle as measured in kilocalories consumed; $t(14) = 0.368$, $p = 0.718$ (Table 3.2 and Figure 3.7). Specifically, within-individual paired raw data did not show significant intra-individual variability in energy intake between kisspeptin and vehicle visits (Figure 3.7). In addition, no significant differences were seen in meal duration ($t(14) = 0.521$, $p = 0.610$) or volume of water drunk while eating ($t(14) = 0.330$, $p = 0.747$) during kisspeptin infusion and vehicle infusion (Table 3.2).

Table 3.2: *Ad libitum* meal parameters during kisspeptin and vehicle infusion

	Vehicle		Kisspeptin		<i>p</i> value
	Mean $\pm$ SEM	95% CI	Mean $\pm$ SEM	95% CI	
Energy intake (kcal)	834.4 $\pm$ 45.1	737.6 - 931.2	844.7 $\pm$ 160.9	755.6 - 933.8	0.718
Water intake (mls)	182.3 $\pm$ 42.6	89.5 - 275.1	161.1 $\pm$ 40.4	73.81 - 248.3	0.747
Meal duration (minutes)	11.3 $\pm$ 0.7	9.8 – 12.8	11.0 $\pm$ 0.9	9.04 – 13.0	0.610

Ad libitum test meal intake parameters showing no significant differences between kisspeptin and vehicle infusion on energy intake, water intake or meal duration, using paired two-tailed t-tests.

Figure 3.7: Energy intake of *ad libitum* meal during kisspeptin and vehicle visits



Energy intake measured in kilocalories; $t(14) = 0.368$, $p = 0.718$, kisspeptin vs vehicle using paired two-tailed t-tests ($N = 15$). Data presented as within-participant paired raw data and mean $\pm$ SEM.

3.5 Discussion

This is the first human study to investigate the effects of peripheral kisspeptin administration on appetite and food intake. In this cohort of healthy men, peripheral kisspeptin administered as a 1 nmol/kg/hour infusion did not acutely affect appetite in the fasted or fed state nor kilocalorie intake during a single *ad libitum* test meal.

Within the animal literature, both central and peripheral kisspeptin administration at bioactive doses can inhibit food intake in male mice (Stengel *et al.*, 2011; Dong *et al.*, 2019). However, the findings are inconsistent in male rats (Thompson *et al.*, 2004; Castellano *et al.*, 2005; Saito *et al.*, 2019), which suggests that the effects of kisspeptin on food intake may be species-specific and affected by methodological differences.

In this study, an intravenous kisspeptin-54 infusion was administered at a dose known to be bioactive in healthy men as demonstrated by increase in plasma kisspeptin levels and increase in serum LH in keeping with previous work (Comninou *et al.*, 2017). Furthermore, peripherally administered kisspeptin-54 is capable of penetrating the blood-brain barrier to reach both GnRH cell bodies and deeper brain structures known to express kisspeptin and its receptor (d'Anglemont de Tassigny *et al.*, 2017; Comninou *et al.*, 2017). Thus, methodologically, the route and dose of administration of kisspeptin used in this study should be adequate for accessing brain pathways involved in regulating appetite and food intake in humans.

In male rodents, the anorectic effects of kisspeptin occur acutely, predominantly within four hours following kisspeptin administration (Stengel *et al.*, 2011; Dong *et al.*, 2019), which encompasses the timeframe of this current study, whereby appetite and food intake were assessed at 30 minutes (pre-meal) and 75 minutes (post-meal) during kisspeptin infusion, when kisspeptin levels are at steady state. Importantly, no significant changes in testosterone were observed between kisspeptin and vehicle infusion during the study, thus removing this

as a confounding factor. Additional efforts were made to reduce sources of variability, and control for potential confounders. All volunteers were male, had similar ages and BMIs, with stable weight for three months prior to enrolment into the study and during the study period. All participants followed the same instructions regarding physical activity, food and caffeine intake prior to each study visit. Furthermore, study visits were performed in a randomised order with participants blinded to the contents of intravenous infusions.

Taken together, these findings suggest that peripheral administration of kisspeptin-54 does not acutely affect appetite or *ad libitum* food intake in healthy men.

3.5.1 Limitations

This study was carried out in healthy eugonadal young men with a narrow BMI range and therefore it may not be generalisable to other populations such as women, as female *Kiss1r* knockout mice display different bodyweight changes and metabolic derangements compared to *Kiss1r* knockout male mice (Tolson *et al.*, 2014; Velasco *et al.*, 2019). It would also be useful to assess the effects of kisspeptin in patients with hypogonadism as kisspeptin improves LH pulsatility in hypogonadotrophic hypogonadism (Jayasena *et al.*, 2009, 2014b), a condition that is associated with derangements in nutritional intake and energy balance (Couzinet *et al.*, 1999) and kisspeptin is able to induce pubertal onset in a rat model of undernutrition (Castellano *et al.*, 2005). Furthermore, hypothalamic Kiss1 expression is reduced in diabetic rats with normalisation of LH and testosterone following repeated kisspeptin administration (Castellano *et al.*, 2006; Dudek *et al.*, 2016), therefore diabetic populations would be another useful cohort in which to assess the effects of kisspeptin on appetite and food intake.

Data from rodent studies indicate that the major anorectic effects of kisspeptin occur during the first four hours, and although this overlaps with the timeframe of my study, there is a possibility that further effects of kisspeptin were not captured as the *ad libitum* meal was given at 45 minutes and the study was completed by 120 minutes. Extending the study period for a

longer duration either as an infusion, or with the use of longer-acting kisspeptin analogues (MacLean *et al.*, 2014) may yield further findings. However, the caveat to this would involve rises in downstream gonadal steroids, which may confound any effects seen as testosterone levels are associated with changes in food intake and dietary patterns in rodents and humans (Nunez & Grundman, 1982; Hu *et al.*, 2018).

3.5.2 Summary

Peripheral administration of kisspeptin-54 at a dose of 1 nmol/kg/hour (which robustly increases LH) does not acutely affect appetite or food intake in healthy eugonadal men. This is the first *in-vivo* human study investigating the effects of kisspeptin on appetite and food intake and contributes to our understanding of kisspeptin's role within reproductive and metabolic pathways in humans.

3.5.3 Future work

As discussed in the limitations above, this is a small cohort study of healthy young men with narrow BMI range and further studies are required to confirm the results and explore the effects of kisspeptin in different groups such as women, and patients with hypogonadal states, or diabetes. Different isoforms of kisspeptin and kisspeptin analogues would also be useful to explore in this context, however kisspeptin-54, as used in this study currently has the most robust evidence base for blood-brain barrier penetrance (d'Anglemont de Tassigny *et al.*, 2017; Comninou *et al.*, 2017) and other isoforms would first need to undergo blood-brain barrier transport studies in order to determine their capacity to reach brain regions controlling appetite and food intake.

As discussed in Chapter 1, the limited body of work detailing the interactions between kisspeptin signalling and metabolic cues has mostly focused on hypothalamic kisspeptin populations. However, kisspeptin and its receptor are also present in numerous other brain

regions, namely within limbic areas that are heavily involved in appetite and food-reward behaviours (Muir *et al.*, 2001; Kotani *et al.*, 2001; Berthoud, 2011). Using fMRI, peripheral kisspeptin administration has been shown to modulate limbic brain pathways associated with sexual and emotional reward processing (Comninou *et al.*, 2017). Therefore, exploration of kisspeptin's effects on brain pathways involved in appetite and food-reward processing using fMRI would provide crucial mechanistic insight into human brain responses to food stimuli. Therefore, in parallel with this study, I have also carried out a fMRI study investigating the effects of kisspeptin on human brain processing in response to food images and the findings are described in the Chapter 4 of this thesis.

Chapter 4

The effects of peripheral kisspeptin administration on brain responses to food images in healthy men.

4.1 Introduction

As discussed in Chapter 3, furthering our knowledge of the links between metabolism and reproduction has major clinical significance, as obesity is a critical public health issue affecting all age groups and populations worldwide (Ritchie & Roser, 2020). In addition to the adverse cardiometabolic outcomes associated with obesity, there is also a significant detrimental impact on reproduction and fertility (Broughton & Moley, 2017; Ramlau-Hansen *et al.*, 2007; Palmer *et al.*, 2012). Kisspeptin is a hypothalamic neuropeptide with a well-characterised reproductive role. (Seminara *et al.*, 2003; Dhillo *et al.*, 2005; Jayasena *et al.*, 2014a). There is also increasing evidence that kisspeptin is involved in central brain pathways controlling appetite and feeding behaviours. Neuroanatomical and functional connections have been described between hypothalamic Kiss1 neurons and the appetite-regulating neuropeptides; NPY and POMC. However, results from *in vitro* and *in vivo* studies differ in regard to the direction and magnitude of kisspeptin's interactions with these pathways (Backholer *et al.*, 2010; Nestor *et al.*, 2016; Fu & Van Den Pol, 2010; Talbi *et al.*, 2016; Cázarez-Márquez *et al.*, 2019).

Global *Kiss1r* knockout mice display metabolic derangements including increased adiposity and body weight changes (Tolson *et al.*, 2014; Velasco *et al.*, 2019). In addition, central and peripheral kisspeptin can reduce food intake in rodents (Stengel *et al.*, 2011; Saito *et al.*, 2019; Dong *et al.*, 2019). Given these observations, and our incomplete understanding of kisspeptin's interactions with central appetite-regulating circuits, further mechanistic studies are required to elucidate the pathways underlying potential metabolic effects of kisspeptin. To date, animal studies in this area have mostly concentrated on hypothalamic kisspeptin signalling (Castellano *et al.*, 2005, 2006; Dudek *et al.*, 2016; Wahab *et al.*, 2011), yet distribution of kisspeptin and its receptor are seen in multiple extra-hypothalamic brain areas with key roles in emotions, appetite and food-reward behaviours (Chapter 1, Table 1.1). Thus

far, the role of kisspeptin signalling in these brain regions has not been explored in relation to human appetite and food-related behaviours.

4.1.1 *Limbic brain regions regulating appetite and food reward*

The hypothalamus is central to the production and integration of hormonal and non-hormonal signals, which contribute to the homeostatic regulation of energy intake i.e. eating when fuels are depleted and abstaining from eating when replete. However, for the vast majority of humans living in a modern energy-rich environment, motivation to eat often exists in the absence of hunger i.e. eating for pleasure. This hedonic drive for eating involves the integration of cognitive, emotional and reward factors through an extensive network of limbic brain regions. This hedonic food network acts in concert with homeostatic pathways in the hypothalamus and brainstem to modulate feelings of hunger, satiety and food-reward behaviours (Berthoud, 2011; Neseliler *et al.*, 2017). The components of this network can be broadly categorised into areas governing sensory processing (insula), evaluative (thalamus), decision making (OFC, ACC), motivational and incentive processing (caudate, globus pallidus, nucleus accumbens, putamen), learning and emotional memory (amygdala and hippocampus) (Figure 4.1).

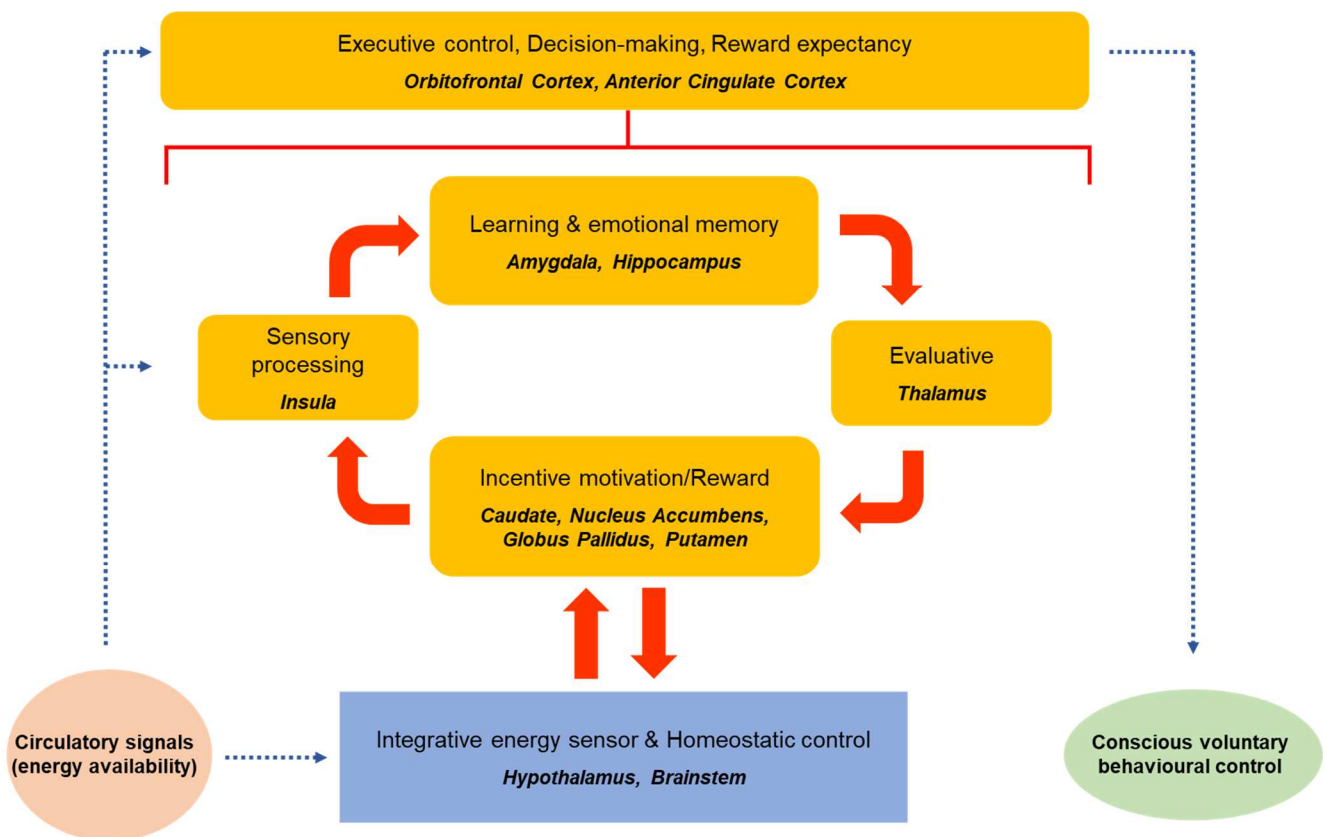
The functional utility of this hedonic food network is demonstrated by studies examining the impact of gut hormones on appetite. Specifically, peripheral signals of satiety including peptide-YY and glucagon-like peptide-1 are associated with reduced activation in the OFC, amygdala and insula along with reduced appetite (Zanchi *et al.*, 2017). In contrast, ghrelin (a peripheral hormone released during fasting, which stimulates appetite and food intake in rodents and humans) (Kojima *et al.*, 1999, Wren *et al.*, 2001a, Wren *et al.*, 2001b) increases neuronal response to food images in the amygdala, OFC, insula and striatum (Berthoud, 2011; Zanchi *et al.*, 2017).

Kisspeptin and its receptor are known to be distributed in these brain areas in humans (Chapter 1, Table 1.1) (Muir *et al.*, 2001; Kotani *et al.*, 2001), and work by Comninou *et al.* demonstrates that peripheral kisspeptin administration can reach limbic regions in healthy men to modulate brain responses to sexual and emotional stimuli (Comninou *et al.*, 2017). Furthermore, my data in Chapter 2 shows that peripheral kisspeptin administration can activate similar hedonic brain pathways in response to an olfactory cue. Consequently, examining the effects of kisspeptin on brain responses to food stimuli could provide key mechanistic insights into the interactions between kisspeptin and food-reward processing in humans.

4.1.2 Summary

There is an expanding body of evidence pointing to a role for the reproductive neuropeptide kisspeptin within appetite regulation pathways. Current mechanistic data is limited to a small number of animal studies that have been primarily focused on hypothalamic kisspeptin signalling, yet kisspeptin and its receptor are also widely distributed in brain regions involved in cognitive, emotional and reward processes that regulate appetite. This is an exciting and highly relevant area that merits exploration to further our understanding of human brain processing in response to food stimuli and the potential interactions between these limbic processes and the kisspeptin system.

Figure 4.1 Schematic representation of the appetitive network in the brain



Schematic representation of the appetitive network in the brain integrating homeostatic and hedonic pathways with circulatory cues and behavioural outputs. It consists of limbic structures involved in sensory processing, evaluative processing, motivation and reward, learning and emotional memory. These structures communicate with homeostatic centres in the hypothalamus and brainstem and feed into executive centres in the orbitofrontal cortex and anterior cingulate cortex that modulate decision making and reward expectancy, ultimately leading to voluntary behavioural control. (Adapted from Berthoud, 2011).

4.2 Aims and hypothesis

Aims:

- To investigate the effects of peripheral kisspeptin administration compared to vehicle on human brain processing in response to food and non-food images by measuring changes in fMRI BOLD activity.
- To correlate kisspeptin-related changes in fMRI BOLD activity with psychometric parameters and hormone profiles.

Hypothesis: Kisspeptin administration in healthy men modulates brain processing in response to viewing food images compared to non-food images.

4.3 Methods

4.3.1 Study approval

The study was performed in accordance with the Declaration of Helsinki. All participants gave written informed consent prior to inclusion in the study. The study was approved by the regional ethics committee (Riverside Research Ethics Committee, London, United Kingdom, REC ref: 17/LO/1504).

4.3.2 Sample size

As this is the first fMRI study to investigate the effects of kisspeptin on brain responses to food images, the sample size was determined based on previously published fMRI studies investigating the effects of other appetite-regulating hormones (peptide-YY, glucagon-like peptide-1, cholecystokinin and ghrelin) using comparable task protocols. These fMRI studies reported significant changes in brain response to food stimuli following a hormonal intervention with sample sizes ranging from 8 to 19 participants (Batterham *et al.*, 2007; Malik *et al.*, 2008; Lassman *et al.*, 2010; De Silva *et al.*, 2011). Therefore, to ensure adequate power to detect significant differences while allowing for natural variation in responses, a sample size of 26 was selected. This is in line with previous work showing significant kisspeptin-enhancement of brain responses to sexual stimuli (Comninou *et al.*, 2017) and compares favourably with empirically derived estimates of optimal sample sizes in fMRI studies, which suggest that sample sizes of 20 to 24 give sufficient power to detect moderate-sized effects (Murphy & Garavan, 2004).

4.3.3 Study recruitment and eligibility criteria

Participants were recruited into the study via print and online advertisements. To confirm eligibility, participants attended a medical screening appointment. Participants were free of current and past physical or psychiatric illness and were naive to psychoactive substances, prescribed or illicit. The Patient Health Questionnaire-9 was used to screen for depression and those scoring above threshold for depressive disorder were excluded from the study (Kroenke *et al.*, 2001). Specific assessment was also undertaken to exclude participants with abnormal eating behaviour using the Dutch Eating Behaviour Questionnaire (van Strien *et al.*, 1986) and the SCOFF questionnaire (Morgan *et al.*, 1999) (Appendix 4). All participants were right-handed, had normal or corrected-to-normal vision and had no contraindications for MRI scanning as listed in MRI safety guidelines (Appendix 1).

4.3.4 Study day protocol

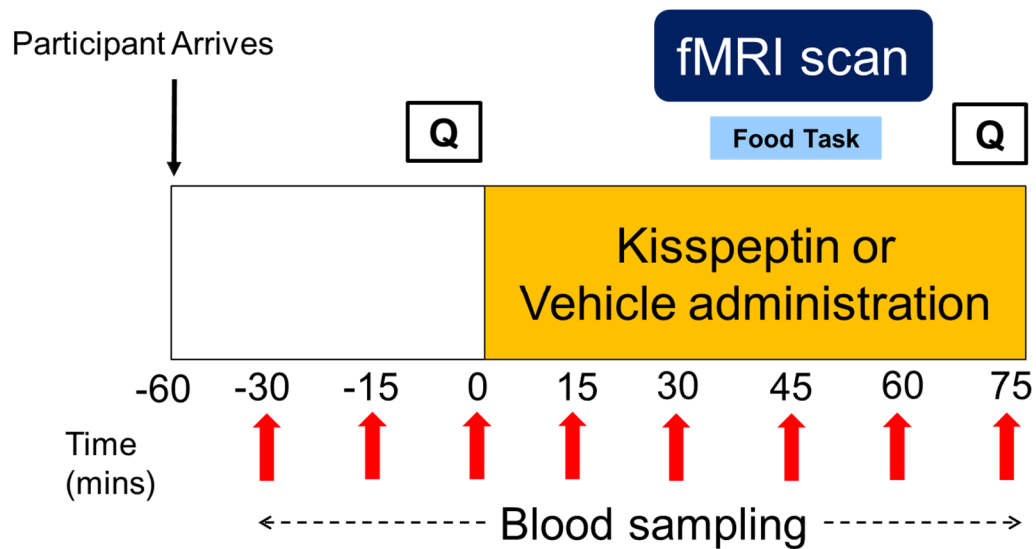
Participants completed two MRI study visits each and were randomised by an independent investigator (using Research Randomizer, www.randomizer.org) to receive either kisspeptin-54 or vehicle at their first visit (Figure 4.2). This was a within-participant design study, in which the participants acted as their own control thereby minimising variability and enhancing power. All study visits commenced in the morning to control for circadian hormonal changes. Participants were instructed to abstain from alcohol and caffeine and to fast from 10pm the night before attending their morning study visit.

On arrival, participants were asked to remove any metal on their person and change into loose hospital scrubs. Intravenous cannulae were inserted into each antecubital fossa for blood collection and infusion of kisspeptin or vehicle. Blood sampling took place at 15 minute intervals from -30 to 75 minutes to measure circulating LH and testosterone levels and to ensure that baseline reproductive hormone levels were equivalent between study visits. At T = 0 minutes, a 75 minute infusion of kisspeptin-54 (1nmol/kg/hour) or vehicle (gelofusine at

equivalent rate) was given. This kisspeptin dose was selected based on previous experience (Comninou 2017), to ensure steady-state levels of circulating kisspeptin from 30 to 75 minutes (during fMRI scanning and tasks) while avoiding increase in downstream testosterone during this timeframe.

Participants completed psychometric questionnaires before and towards the end of the kisspeptin or vehicle infusion as detailed below. fMRI tasks were initiated at 30 minutes from start of infusion to allow plasma kisspeptin levels to reach steady state.

Figure 4.2: Study day protocol



Participants attended for two study visits each, one for intravenous administration of kisspeptin (1 nmol/kg/hour) and one for intravenous administration of an equivalent volume of vehicle for 75 minutes. Participants completed baseline and intra-infusion questionnaires (Q) and blood sampling took place at time-points -30, -15, 0, 15, 30, 45, 60, and 75 minutes to measure circulating LH and testosterone during the study. Infusion commenced at T = 0 minutes and at T = 30 minutes, when kisspeptin levels reached steady state, participants underwent functional MRI scanning while performing a food task.

4.3.5 Kisspeptin-54 peptide preparation and infusion

Kisspeptin-54 was prepared using the same protocol as Chapters 2 and 3. In brief, human sequence kisspeptin-54 peptide was freeze-dried and stored at -20°C. Vials were defrosted on the morning of the study and reconstituted in 0.9% saline prior to administration. Kisspeptin-54 was infused at a rate of 1 nmol/kg/hour, a dose known to significantly increase LH levels in men (Comninou *et al.*, 2017). Vehicle infusions consisted of gelofusine (Braun, Germany), administered at an equivalent rate to the kisspeptin infusion for each participant.

4.3.6 Blood sample collection and analyses

Blood samples were collected at the timepoints shown in Figure 4.2. Blood samples for LH and testosterone measurement were collected into tubes containing clot activator (BD Vacutainer). After collection, blood samples were left to clot at room temperature for ≥ 30 minutes and then centrifuged at 3000 RPM for 10 minutes at room temperature. Serum was separated and transferred into LP4 tubes. All samples were stored immediately at -20°C then defrosted prior to sample analysis.

Circulating LH and testosterone levels were measured using automated chemiluminescent immunoassays (Abbott Diagnostics, Maidenhead, UK). The intra-assay and inter-assay coefficients of variation were $< 5\%$ for LH and testosterone. Analytical sensitivities were: 0.5 IU/L (LH) and 2 nmol/L (testosterone). Limits of detectability for each assay were: LH, 0.07 IU/L; and testosterone, 4.9 nmol/L.

4.3.7 fMRI procedure

The principles of fMRI study design, data acquisition and analysis have been discussed in Chapter 2. This study involved a similar fMRI procedure whereby a series of anatomical and functional brain scans were performed. For the functional task a mirror mounted on the head coil was used to view a screen at the rear of the scanner bore onto which the stimulus images

were projected. To record task responses, participants were equipped with a custom-made five button MRI-compatible response box. In addition, a pulse-oximeter was used to monitor physiological data by means of a standard data-recording system (AD Instruments PowerLab) in the control room. Kisspeptin and vehicle infusions were administered via a Medrad Spectris Solaris MRI compatible injection system controlled from a remote panel in the control room.

4.3.8 Food task

In keeping with previously published protocols, a block-design was employed for this task in which participants were presented with images of high calorie foods, low calorie foods and neutral non-food images as a control (Figure 4.3) (Beaver *et al.*, 2006; De Silva *et al.*, 2011). All images were selected from a database of high quality standardised food and non-food images that have been validated across several European countries by independent adult raters (Charbonnier *et al.*, 2015). Blocks of high calorie food, low calorie food and neutral non-food images were presented sequentially in blocks of four images. Each image was displayed for 4 seconds; each block was therefore 16 seconds in duration. Resting blocks in which a grey screen with a central fixation cross was displayed were also included in order to provide a baseline for subsequent analysis. There were eight blocks of each type (high calorie, low calorie, neutral, and rest), with 32 blocks in total. Blocks were presented in a pre-determined pseudo-random order where the order of blocks was randomised within meta-blocks of four (one of each type), and with the constraint that the same block type could not be presented successively (i.e. at the end of one meta-block, and the beginning of the next). Total task duration was 8 minutes and 32 seconds, plus a 10 second buffer period at the end of the task. To maintain alertness and task engagement, participants were asked to rate the pleasantness of each image on a five-point scale ranging from “very unpleasant” to “very pleasant” using the five-button response box.

Figure 4.3 Examples of standardised images used during the food task

High calorie food



Low calorie food



Neutral non-food



Sample images of high calorie food, low calorie food and neutral non-food items from a database of standardised images (Charbonnier *et al.*, 2015). All items were displayed on identical backgrounds to remove other visual confounders during the task.

4.1.1 Selecting regions of interest

A set of *a priori* selected brain regions defined in standard stereotactic space using the Harvard-Oxford atlases (<https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/>), was used to extract data for ROI analyses. A total of 10 regions were selected as brain areas involved in food-reward pathways that also express kisspeptin receptors in humans. These included the amygdala, ACC, caudate, globus pallidus, hippocampus, insula, nucleus accumbens, putamen, OFC and thalamus (Batterham *et al.*, 2007; De Silva *et al.*, 2011; Zanchi *et al.*, 2017; Beaver *et al.*, 2006; Malik *et al.*, 2008). Additionally, a composite “food-reward system” mask was derived using meta-analytic data within the Neurosynth database (<http://neurosynth.org/>) to assess system-based effects of kisspeptin compared to vehicle during the food task.

4.1.2 Psychometric questionnaires

Trait questionnaires

At their first study visit, participants were asked to complete a set of psychometric questionnaires to assess certain baseline traits prior to commencing the infusion and starting the MRI scan (Appendix 7). The 20-item State-Trait Anxiety Inventory (Okun *et al.*, 1996) was used to record longstanding trait anxiety levels. Subjective perceptions of happiness and general satisfaction with life were measured using the Subjective Happiness Scale (Lyubomirsky & Lepper, 1999) and the Satisfaction with Life Scale (Diener *et al.*, 1985) respectively. The 18-item Three-Factor Eating Questionnaire was used to collect data on eating traits including cognitive restraint, uncontrolled eating and emotional eating (Blandine de Lauzon *et al.*, 2004). The 24-item Behavioral Inhibition and Activation System Scales (BIS/BAS) (Carver C.S, 1994) was used to assess tendency towards anticipation of punishment (BIS) and to reward (BAS); traits which have been shown to predict fMRI brain responses to appetising food images (Beaver *et al.*, 2006).

State questionnaires

A second set of questionnaires were used to assess food craving, appetite and satiety parameters in the current moment (Appendix 8). These included visual analog scales (VAS) as used in Chapter 3 and the Food Cravings Questionnaire (FCQ). A 10 cm VAS scale was used to record responses to six questions assessing hunger, satiety, nausea and sleepiness. The FCQ is a 15-item questionnaire that has been validated for the assessment of hunger in the general population (Nijs *et al.*, 2007; Cepeda-Benito *et al.*, 2000) and is widely used in appetite studies (Rebello & Greenway, 2016; Meule, 2018). It explores food cravings using five domains; an intense desire to eat; anticipation of positive reinforcement that may result from eating; anticipation of relief from negative states as a result of eating; a lack of control over eating; and hunger as a physiological state. Each participant was asked to complete these questionnaires before and towards the end of infusion (kisspeptin or vehicle) on their first and second study visits.

4.1.3 Statistical analyses

Statistical analyses were performed using GraphPad Prism 8 software. Data were normally distributed by Kolmogorov testing. Hormone level data analysis was performed using two-way repeated measures ANOVA with mixed effects modelling. Paired two-tailed t-tests were performed to investigate the kisspeptin effect on each individual ROI, with an α threshold of $p < 0.05$ determining statistical significance.

ROI data were also examined using a test of equivalence to provide additional support for an absence of effect (Lakens, 2017). The widely accepted two one-sided t-test (TOST) was chosen for this study in which the null hypothesis was expressed as “kisspeptin has no effect on food-reward brain regions such as X”. In the TOST procedure the null hypothesis is rejected when the effect sits within the predetermined lower and upper equivalence bounds. If this is the case, then it can be assumed that the effect is close enough to zero to be considered a true absence of effect (Lakens, 2013, 2017).

In this study the TOST procedure was performed using an openly available statistical package provided by Lakens *et al* for the Jamovi ® statistical software. The equivalence bounds were pre-determined using Cohen's d of -0.5 to 0.5 as a standardised mean difference constituting a medium effect size (Lakens, 2013).

4.2 Results

4.2.1 Baseline participant characteristics

In total, 31 participants took part in the study. Two participants did not complete both study visits and two participants were excluded due to technical failure, giving a final study group of 27 healthy men aged between 18 and 36 years. Baseline trait questionnaires confirmed that all the participants had normal baseline characteristics for anxiety, mood, and eating behaviours (Table 4.1).

4.2.2 Effects of kisspeptin administration on circulating hormone levels

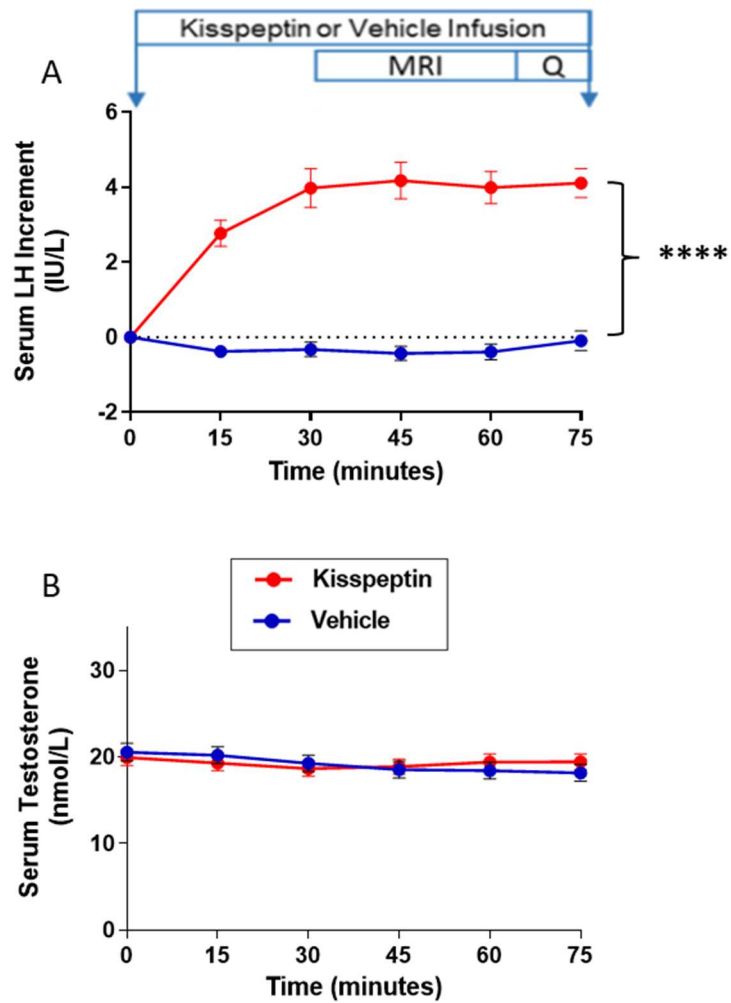
Intravenous kisspeptin infusion (1 nmol/kg/hour) significantly increased serum LH levels (Figure 4.4A), which confirmed that the dose of kisspeptin used was biologically active. In keeping with the literature and my findings in Chapter 2, kisspeptin administration had no significant effects on testosterone levels during the 75 minute study period (Figure 4.4B), which has previously been shown to occur following longer periods of kisspeptin exposure in humans (Jayasena *et al.*, 2011).

Table 4.1: Participant clinical and psychometric characteristics

		Healthy Men (N = 27)
		Mean $\pm$ SEM
Age (years)		26.5 $\pm$ 1.1
BMI (kg/m²)		23.9 $\pm$ 0.4
Baseline Reproductive Hormones		
	LH (IU/L)	2.6 $\pm$ 0.2
	Testosterone (nmol/L)	20.4 $\pm$ 0.9
STAI-Y Trait		36.6 $\pm$ 1.8
SwLS		24.9 $\pm$ 1.1
SHS		17.7 $\pm$ 0.5
BIS		19.4 $\pm$ 0.8
BAS		
	Drive	11.6 $\pm$ 0.4
	Fun	12.2 $\pm$ 0.5
	Reward	17.5 $\pm$ 0.4
TFEQ		
	Cognitive Restraint	24.2 $\pm$ 2.3
	Uncontrolled Eating	31.4 $\pm$ 2.5
	Emotional Eating	22.8 $\pm$ 3.3
	Total Score	27.6 $\pm$ 1.9

BMI = Body Mass Index; LH = Luteinising Hormone; STAI-Y Trait = State-Trait Anxiety Inventory to assess trait anxiety; SwLS = Satisfaction with Life Scale to assess satisfaction with life as a whole; SHS = Subjective Happiness Scale; BIS = Behavioral Inhibition System Scale to assess sensitivity to anticipation of punishment; BAS = Behavioral Activation System Scale to assess sensitivity to desired goals, fun and reward; TFEQ = Three Factor Eating Questionnaire to assess trait eating behaviours. Results confirmed normal baseline hormone levels, no anxiety trait and no abnormal eating traits. Data presented as mean $\pm$ SEM.

Figure 4.4: Effects of peripheral kisspeptin administration on circulating hormone levels



A: Kisspeptin infusion significantly increased circulating levels of LH ($p < 0.0001$) compared to vehicle.

B: Kisspeptin had no effect on circulating testosterone levels during the same timeframe.

Data depict mean $\pm$ SEM, **** $p < 0.0001$ using two-way repeated measures ANOVA with mixed effects modelling (N = 27).

4.2.3 Effects of kisspeptin administration on state measures of appetite during kisspeptin and vehicle visits.

Changes in appetite during each study visit were assessed using the FCQ and VAS questions. Baseline FCQ and VAS scores were equivalent across all domains on both study days (Table 4.2). Comparable with my findings in Chapter 3, this study also found no significant changes in FCQ or VAS parameters during kisspeptin administration compared to vehicle (Table 4.2, Figures 4.5 and 4.6).

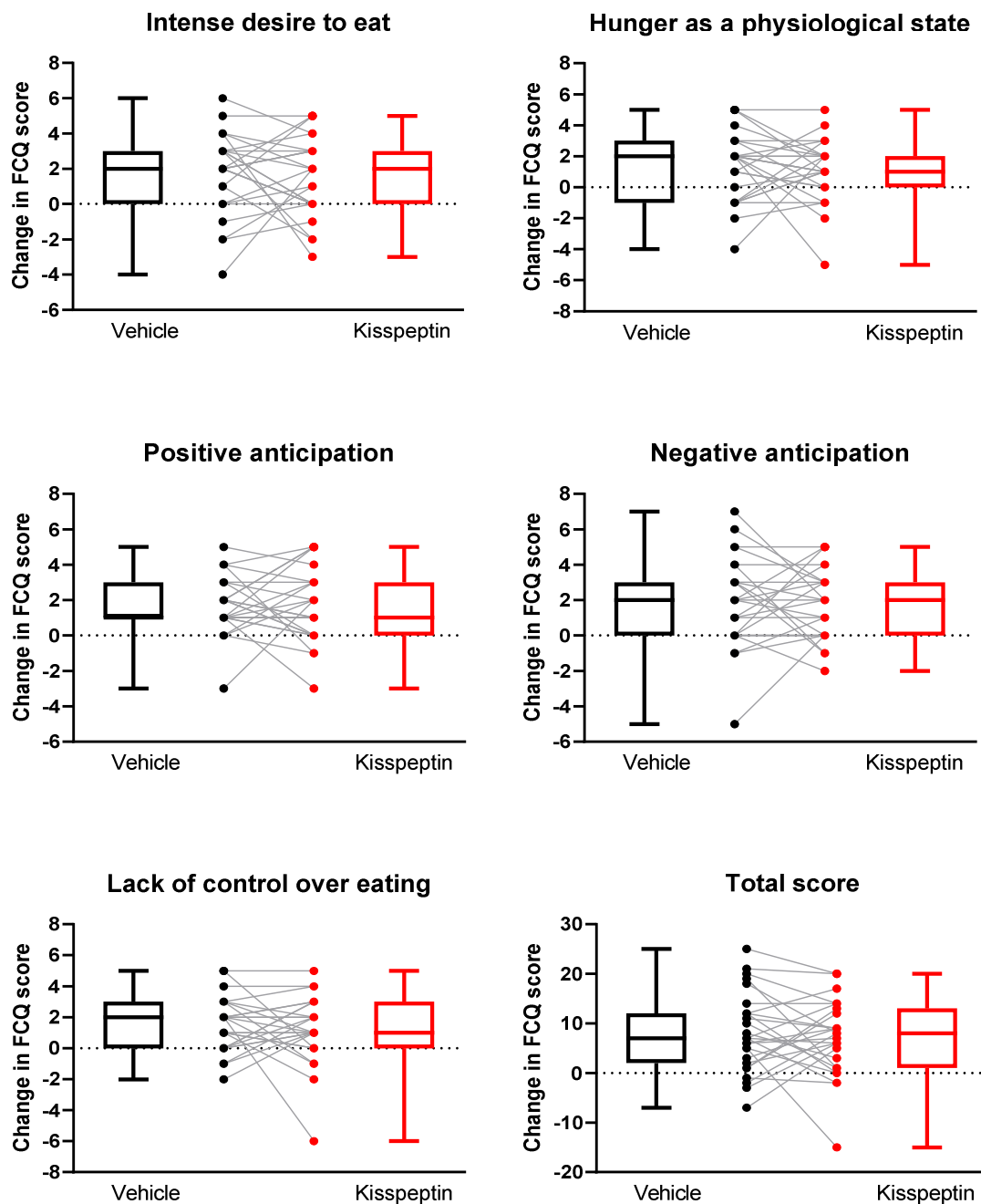
Reassuringly, there were also no significant differences in other confounding factors such as nausea ($t(26) = 0.588$, $p = 0.561$) or sleepiness ($t(26) = 0.215$, $p = 0.831$) during kisspeptin compared to vehicle administration (Table 4.2).

Table 4.2: Baseline and change in state psychometric characteristics

	Kisspeptin Visit	Vehicle Visit
	Mean $\pm$ SEM	Mean $\pm$ SEM
Baseline FCQ scores		
Intense desire to eat	8.8 $\pm$ 0.6	8.3 $\pm$ 0.6
Anticipation of positive reinforcement	8.7 $\pm$ 0.5	8.3 $\pm$ 0.5
Anticipation of relief from negative states	9.1 $\pm$ 0.5	8.3 $\pm$ 0.5
Lack of control over eating	6.0 $\pm$ 0.6	5.9 $\pm$ 0.5
Hunger as a physiological state	9.4 $\pm$ 0.5	9.2 $\pm$ 0.6
Total score	42.0 $\pm$ 2.1	40.1 $\pm$ 2.4
Change in FCQ scores		
Intense desire to eat	1.5 $\pm$ 0.4	1.8 $\pm$ 0.4
Anticipation of positive reinforcement	1.7 $\pm$ 0.4	1.7 $\pm$ 0.4
Anticipation of relief from negative states	1.7 $\pm$ 0.4	1.7 $\pm$ 0.5
Lack of control over eating	1.2 $\pm$ 0.4	1.5 $\pm$ 0.3
Hunger as a physiological state	1.1 $\pm$ 0.4	1.4 $\pm$ 0.4
Total score	7.1 $\pm$ 1.5	8.1 $\pm$ 1.5
Baseline VAS scores		
How hungry are you right now	4.9 $\pm$ 0.5	4.9 $\pm$ 0.5
How pleasant would it be to eat right now	6.0 $\pm$ 2.0	5.6 $\pm$ 0.5
How much could you eat right now	6.2 $\pm$ 0.4	6.0 $\pm$ 0.5
How full do you feel right now	3.2 $\pm$ 0.4	3.1 $\pm$ 0.5
How nauseous do you feel right now	1.3 $\pm$ 0.3	1.2 $\pm$ 0.3
How sleepy do you feel right now	5.0 $\pm$ 0.5	4.8 $\pm$ 0.5
Change in VAS scores		
How hungry are you right now	1.7 $\pm$ 0.3	1.6 $\pm$ 0.3
How pleasant would it be to eat right now	1.5 $\pm$ 0.3	1.4 $\pm$ 0.3
How much could you eat right now	1.1 $\pm$ 0.2	1.0 $\pm$ 0.3
How full do you feel right now	-0.6 $\pm$ 0.3	-0.7 $\pm$ 0.4
How nauseous do you feel right now	-0.3 $\pm$ 0.2	-0.1 $\pm$ 0.3
How sleepy do you feel right now	1.4 $\pm$ 0.5	1.2 $\pm$ 1.9

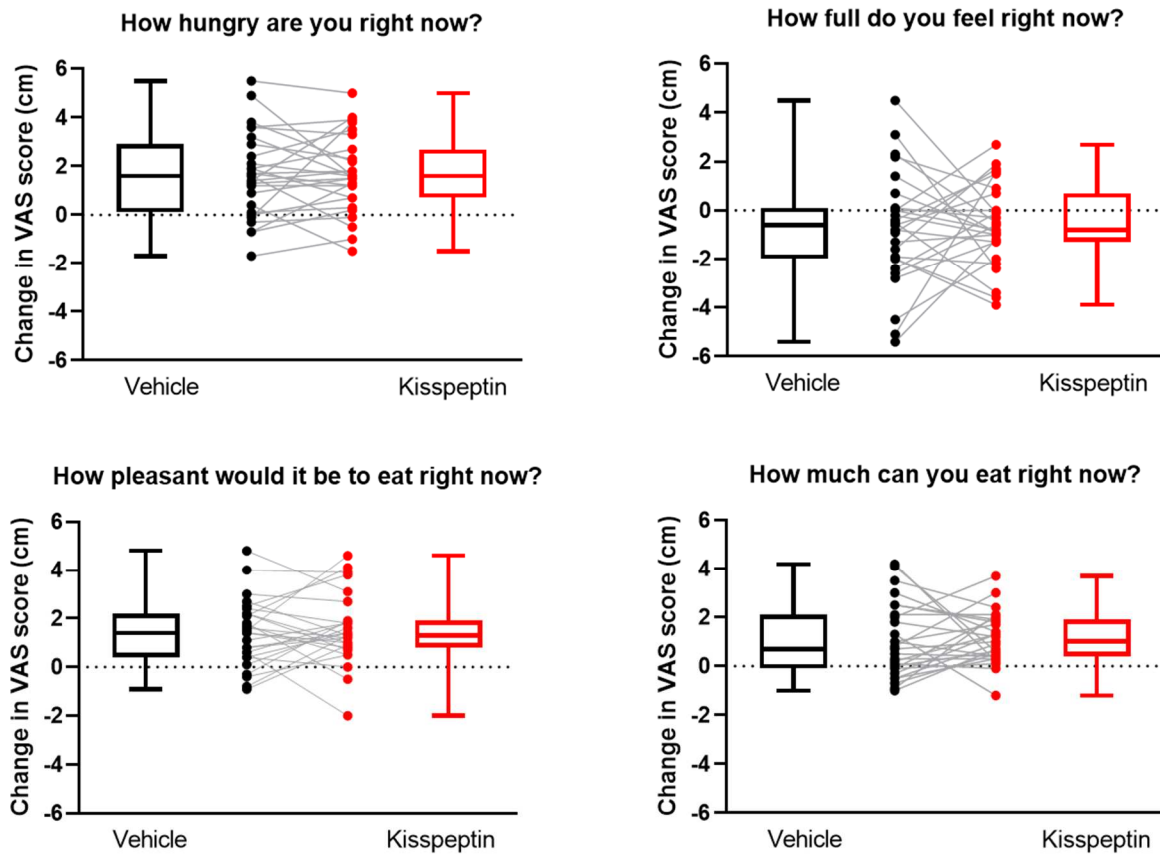
FCQ = food cravings questionnaire; VAS = visual analog scale. Change in scores calculated as the difference between pre-infusion scores and end-of-infusion scores. N = 13 participants received vehicle and N = 14 received kisspeptin at first visit. No significant difference in parameters between visits assessed by paired two-tailed t-tests.

Figure 4.5: Change in FCQ parameters during kisspeptin and vehicle administration



FCQ = food cravings questionnaire. Intense desire to eat: $t(26) = 0.553$, $p = 0.585$; hunger as a physiological state: $t(26) = 0.631$, $p = 0.534$; positive anticipation: $t(26) = 0$, $p > 0.999$; negative anticipation: $t(26) = 0.147$, $p = 0.884$; lack of control over eating: $t(26) = 0.589$, $p = 0.561$; total score: $t(26) = 0.599$, $p = 0.554$. Paired two-tailed t-tests. Data presented as within-participant paired raw data and box and whiskers plot depicting maximum, minimum and mean values ($N = 27$).

Figure 4.6: Change in VAS appetite parameters during kisspeptin and vehicle administration



VAS = visual analog scale. how hungry are you right now: $t(26) = 0.078$, $p = 0.938$; how full do you feel right now: $t(26) = 0.294$, $p = 0.771$; how pleasant would it be to eat right now: $t(26) = 0.533$, $p = 0.599$; how much could you eat right now: $t(26) = 0.358$, $p = 0.724$. Paired two-tailed t-tests. Data presented as within-participant paired raw data and box and whiskers plot depicting maximum, minimum and mean values (N = 27).

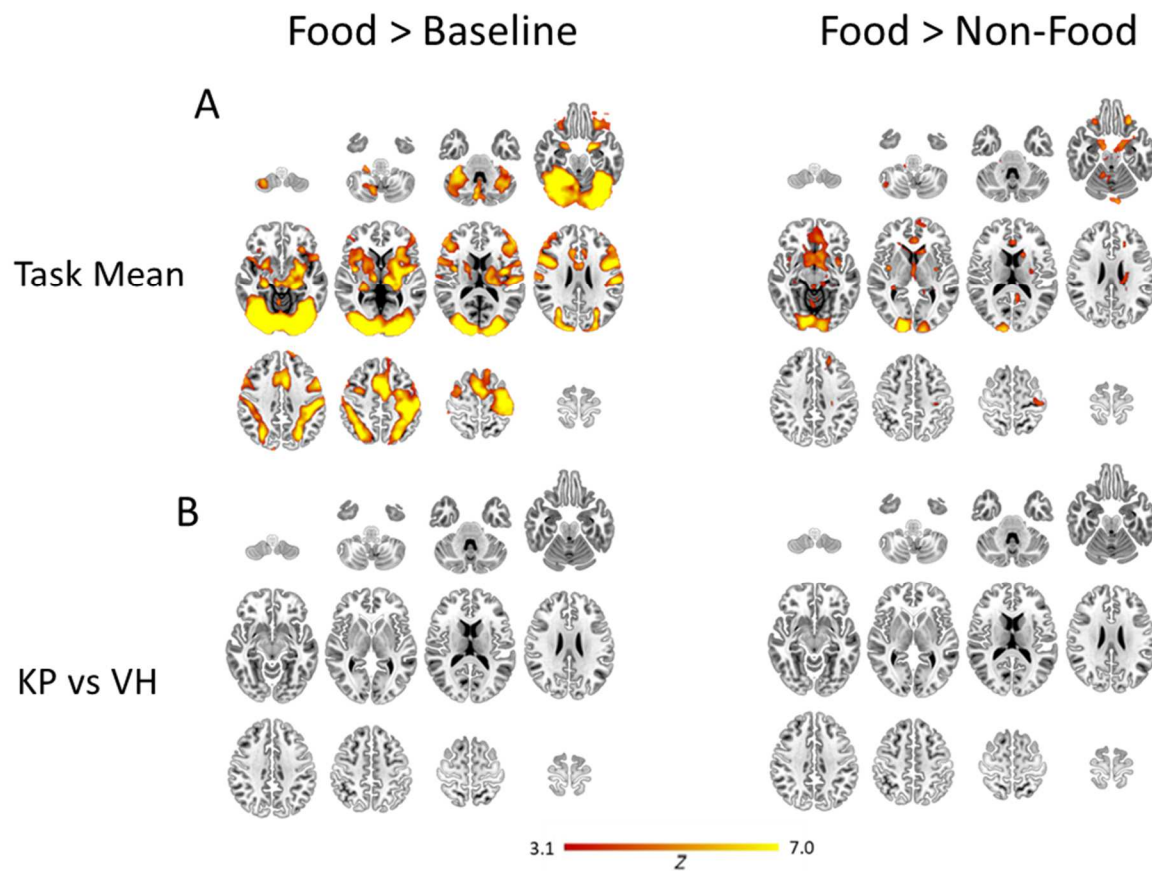
4.2.4 Food task results

Whole brain voxel-wise analysis

Group-mean analysis of the food image trials for all participants and both study visits showed increased activity in key limbic regions involved in hedonic food processing, thus confirming that the food images worked effectively compared to neutral non-food images and baseline (derived from BOLD activity during rest periods when a blank grey screen was displayed) (Figure 4.7A). When food images were sub-divided into high calorie and low calorie foods, group-mean analysis for the respective image trials also confirmed that both high calorie and low calorie foods enhanced BOLD activity when compared to neutral non-food images (Figure 4.8A). Additionally, high calorie foods evoked enhanced BOLD activity when compared to low calorie foods as expected (Figure 4.8A).

Subsequently, a treatment comparison analysis was carried out on a whole brain voxel-wise level to compare differences in brain response between kisspeptin and vehicle visits. This showed no differences in brain activity during kisspeptin infusion compared to vehicle for any of the image contrasts that were tested (Figures 4.7B and 4.8B).

Figure 4.7: Task means and treatment effects for all food images

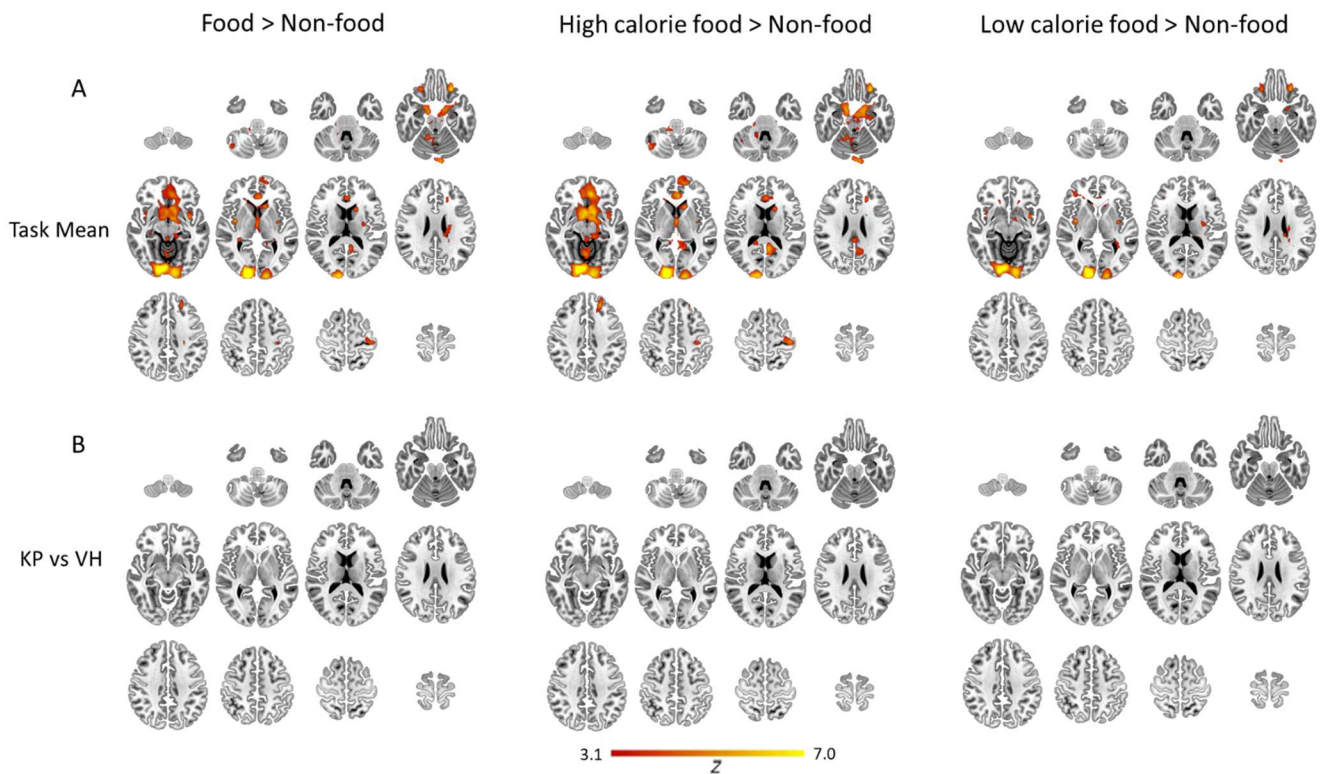


A: Group mean analysis results for the main effects of the food image trials in all participants and both treatments (kisspeptin and vehicle) relative to baseline.

B: Within-subject whole-brain analysis of the effects of kisspeptin (KP) vs. vehicle (VH) for food images against baseline and food images against non-food images.

Positive voxel values (red-yellow) represent an increase in BOLD activity. All statistical maps thresholded at $z = 3.1$, $p < 0.05$ (cluster corrected for multiple comparisons).

Figure 4.8: Task means and treatment effects for high calorie and low calorie food images



A: Group mean analysis results for the main effects of high calorie food against non-food, low calorie food against non-food and high calorie food against low calorie food image trials relative to baseline in all participants and both treatments (kisspeptin and vehicle).

B: Within-subject whole-brain analysis showing no effects of kisspeptin (KP) vs vehicle (VH) for high calorie food against non-food, low calorie food against non-food and high calorie food against low calorie food relative to baseline image trials.

Positive voxel values (red-yellow) represent an increase in BOLD activity. All statistical maps thresholded at $z = 3.1$, $p < 0.05$ (cluster corrected for multiple comparisons).

Regions of interest analysis

Paired two-tailed t-tests were performed for the set of 10 *a priori* defined anatomical regions; amygdala, ACC, caudate, globus pallidus, hippocampus, insula, nucleus accumbens, putamen, OFC and thalamus. The results showed no significant differences between kisspeptin and vehicle in any of the ROIs examined. To fully assess the effects of kisspeptin compared to vehicle on ROI BOLD activity during the food task, several different image contrasts were investigated, with no significant results observed. These are described as follows:

High calorie food against baseline (Table 4.3)

Low calorie food against baseline (Table 4.4)

Neutral non-food against baseline (Table 4.5)

All food against neutral non-food images (Table 4.6)

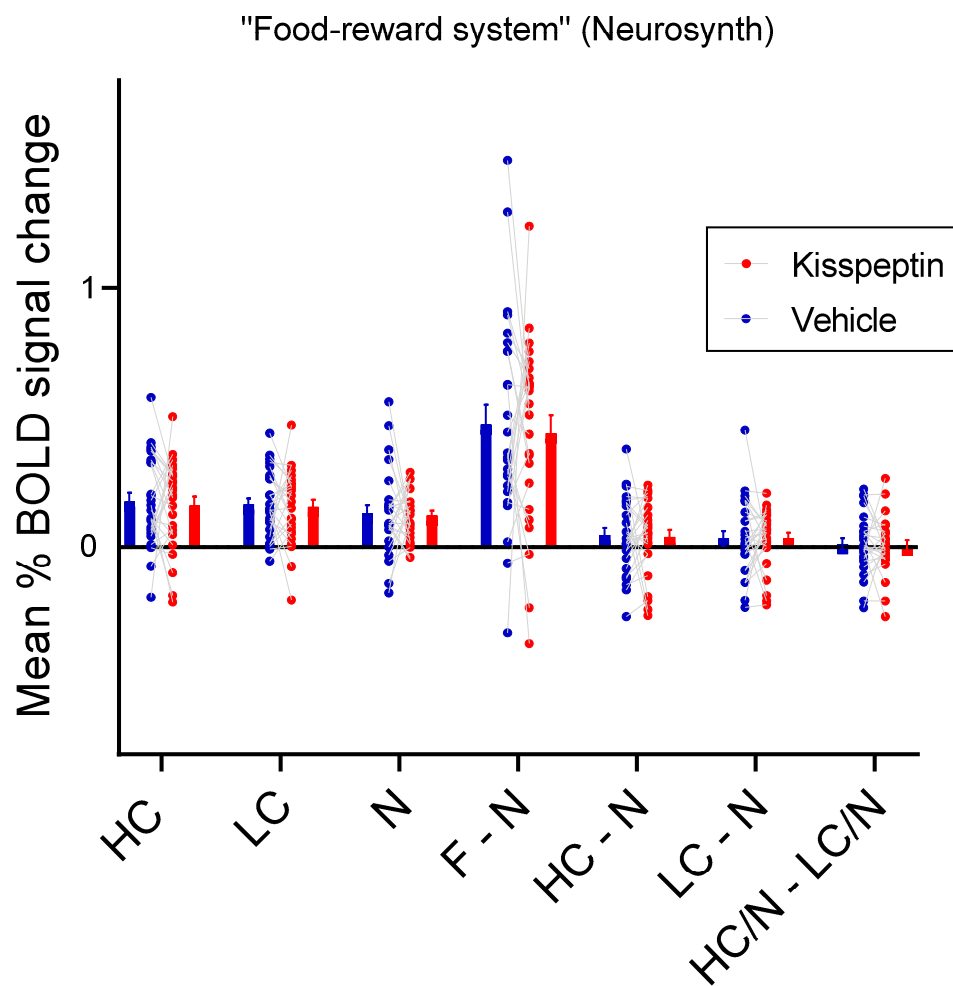
High calorie food against neutral non-food images (Table 4.7)

Low calorie food against neutral non-food images (Table 4.8)

High calorie food against low calorie food (where changes in BOLD activity during neutral non-food images have been subtracted from both sets of stimuli) (Table 4.9)

The global lack of kisspeptin-effect during the food task was also illustrated on a systems-based ROI analysis using a “food-reward” brain mask derived from meta-analytic data within the Neurosynth database (Yarkoni *et al.*, 2011) (Figure 4.9 and Tables 4.3 to 4.9).

Figure 4.9: Systems-based ROI analysis of kisspeptin vs vehicle effects during the food task



Effects of kisspeptin compared to vehicle on %BOLD signal change in the Neurosynth-derived “food-reward system” mask.

HC = high calorie food (against baseline): $t(26) = 0.355$, $p = 0.736$; LC = low calorie food (against baseline): $t(26) = 0.251$, $p = 0.804$; N = neutral non-food (against baseline): $t(26) = 0.227$, $p = 0.822$; F-N = all food against neutral non-food: $t(26) = 0.317$, $p = 0.754$; HC-N = high calorie against neutral non-food: $t(26) = 0.185$, $p = 0.855$; LC-N = low calorie against neutral non-food: $t(26) = 0.027$, $p = 0.979$; HC/N – LC/N = high calorie against low calorie food (subtracting neutral images from both food stimuli): $t(26) = 0.313$, $p = 0.757$.

Data depict within participant paired raw data, mean $\pm$ SEM. Paired two-tailed t-tests ($N = 27$).

TOST equivalence tests

Further analysis was performed using the TOST equivalence test to determine whether the non-significant results observed at the ROI level represented insufficient power of the study or if the effect size was small enough to be considered a true negative. TOST equivalence tests were carried out in tandem with paired two-tailed t-tests for all the image contrasts assessed in the ROI analyses (Tables 4.3 to 4.9). Pre-determined equivalence bounds were selected as Cohen's d of -0.5 (lower bound) and 0.5 (upper bound). Two p values were generated per TOST; one at the upper bound and one at the lower bound. The results demonstrated that kisspeptin's effects on all ROIs in all contrasts were significantly within both upper and lower equivalence bounds. Therefore, the effect size could be considered close enough to zero for the null hypothesis of a lack of effect to be accepted. In keeping with conventional reporting of TOST equivalence tests (Lakens, 2017), the less significant (larger) p value is stated for each TOST result in Tables 4.3 to 4.9.

Collectively, these data indicate that kisspeptin does not modulate fMRI brain response to food imagery in healthy men.

Table 4.3: Effects of kisspeptin compared to vehicle in *a priori* defined anatomical ROIs for high calorie food against baseline

Kisspeptin	Vehicle		t	df	p
ACC (HC)	ACC (HC)	t-test	-0.844	26	0.406
		TOST	1.75	26	0.046
Accumbens (HC)	Accumbens (HC)	t-test	0.152	26	0.881
		TOST	-2.45	26	0.011
Amygdala (HC)	Amygdala (HC)	t-test	-0.203	26	0.840
		TOST	2.39	26	0.012
Caudate (HC)	Caudate (HC)	t-test	-0.133	26	0.895
		TOST	2.47	26	0.010
Globus Pallidus (HC)	Globus Pallidus (HC)	t-test	-0.005	26	0.996
		TOST	2.59	26	0.008
Hippo (HC)	Hippo (HC)	t-test	-0.272	26	0.788
		TOST	2.33	26	0.014
Insula (HC)	Insula (HC)	t-test	-0.127	26	0.900
		TOST	2.47	26	0.010
OFC (HC)	OFC (HC)	t-test	-0.384	26	0.705
		TOST	2.21	26	0.018
Putamen (HC)	Putamen (HC)	t-test	0.180	26	0.858
		TOST	-2.42	26	0.011
Thalamus (HC)	Thalamus (HC)	t-test	0.688	26	0.497
		TOST	-1.91	26	0.034
“Food reward” mask (HC)	“Food reward” mask (HC)	t-test	-0.355	26	0.726
		TOST	2.24	26	0.017

HC = high calorie food against baseline. Data presented as paired two-tailed t-tests and TOST (two one-sided t-tests) with upper and lower equivalence bounds set as Cohen's d -0.5 and 0.5. TOST generates p values for the upper equivalence bound and the lower equivalence bound, the less significant p value is presented in the table above.

Table 4.4: Effects of kisspeptin compared to vehicle in *a priori* defined anatomical ROIs for low calorie food against baseline

Kisspeptin	Vehicle		t	df	p
ACC (LC)	ACC (LC)	t-test	-0.676	26	0.505
		TOST	1.92	26	0.033
Accumbens (LC)	Accumbens (LC)	t-test	0.615	26	0.544
		TOST	-1.98	26	0.029
Amygdala (LC)	Amygdala (LC)	t-test	-0.436	26	0.666
		TOST	2.16	26	0.020
Caudate (LC)	Caudate (LC)	t-test	0.349	26	0.730
		TOST	-2.25	26	0.017
Globus Pallidus (LC)	Globus Pallidus (LC)	t-test	0.235	26	0.816
		TOST	-2.36	26	0.013
Hippocampus (LC)	Hippocampus (LC)	t-test	0.168	26	0.868
		TOST	-2.43	26	0.011
Insula (LC)	Insula (LC)	t-test	-0.097	26	0.924
		TOST	2.50	26	0.009
OFC (LC)	OFC (LC)	t-test	0.520	26	0.608
		TOST	-2.08	26	0.024
Putamen (LC)	Putamen (LC)	t-test	0.017	26	0.987
		TOST	-2.58	26	0.008
Thalamus (LC)	Thalamus (LC)	t-test	1.018	26	0.318
		TOST	-1.58	26	0.063
“Food reward” mask (LC)	“Food reward” mask (LC)	t-test	-0.251	26	0.804
		TOST	2.35	26	0.013

LC = low calorie food against baseline. Data presented as paired two-tailed t-tests and TOST (two one-sided t-tests) with upper and lower equivalence bounds set as Cohen's d -0.5 and 0.5. TOST generates p values for the upper equivalence bound and the lower equivalence bound, the less significant p value is presented in the table above.

Table 4.5: Effects of kisspeptin compared to vehicle in *a priori* defined anatomical ROIs for neutral non-food images against baseline

Kisspeptin	Vehicle		t	df	p
ACC (N)	ACC (N)	t-test	-0.805	26	0.428
		TOST	1.79	26	0.042
Accumbens (N)	Accumbens (N)	t-test	0.138	26	0.891
		TOST	-2.46	26	0.010
Amygdala (N)	Amygdala (N)	t-test	0.005	26	0.996
		TOST	-2.59	26	0.008
Caudate (N)	Caudate (N)	t-test	0.389	26	0.700
		TOST	-2.21	26	0.018
Globus Pallidus (N)	Globus Pallidus (N)	t-test	-0.021	26	0.984
		TOST	2.58	26	0.008
Hippocampus (N)	Hippocampus (N)	t-test	0.170	26	0.866
		TOST	-2.43	26	0.011
Insula (N)	Insula (N)	t-test	-0.097	26	0.924
		TOST	2.50	26	0.009
OFC (N)	OFC (N)	t-test	-0.227	26	0.822
		TOST	2.37	26	0.013
Putamen (N)	Putamen (N)	t-test	0.218	26	0.829
		TOST	-2.38	26	0.012
Thalamus (N)	Thalamus (N)	t-test	0.872	26	0.391
		TOST	-1.73	26	0.048
“Food reward” mask (N)	“Food reward” mask (N)	t-test	-0.227	26	0.822
		TOST	2.37	26	0.013

N = neutral non-food images against baseline. Data presented as paired two-tailed t-tests and TOST (two one-sided t-tests) with upper and lower equivalence bounds set as Cohen’s d -0.5 and 0.5. TOST generates p values for the upper equivalence bound and the lower equivalence bound, the less significant p value is presented in the table above.

Table 4.6: Effects of kisspeptin compared to vehicle in *a priori* defined anatomical ROIs for all food against neutral non-food images

Kisspeptin	Vehicle		t	df	p
Accumbens (F- N)	Accumbens (F- N)	t-test	0.339	26	0.737
		TOST	-2.26	26	0.016
Amygdala (F- N)	Amygdala (F- N)	t-test	-0.220	26	0.828
		TOST	2.38	26	0.013
ACC (F- N)	ACC (F- N)	t-test	-0.888	26	0.383
		TOST	1.71	26	0.050
Caudate (F- N)	Caudate (F- N)	t-test	0.189	26	0.852
		TOST	-2.41	26	0.012
Globus Pallidus (F- N)	Globus Pallidus (F- N)	t-test	0.066	26	0.948
		TOST	-2.53	26	0.009
Hippocampus (F- N)	Hippocampus (F- N)	t-test	0.031	26	0.976
		TOST	-2.57	26	0.008
Insula (F- N)	Insula (F- N)	t-test	-0.122	26	0.904
		TOST	2.48	26	0.010
Putamen (F- N)	Putamen (F- N)	t-test	0.001	26	1.000
		TOST	-2.60	26	0.008
Thalamus (F- N)	Thalamus (F- N)	t-test	0.953	26	0.349
		TOST	3.55	26	0.001
"Food reward" mask (F- N)	"Food reward" mask (F- N)	t-test	-0.317	26	0.754
		TOST	2.28	26	0.015

F-N = all food against neutral non-food images. Data presented as paired two-tailed t-tests and TOST (two one-sided t-tests) with upper and lower equivalence bounds set as Cohen's d -0.5 and 0.5. TOST generates p values for the upper equivalence bound and the lower equivalence bound, the less significant p value is presented in the table above.

Table 4.7: Effects of kisspeptin compared to vehicle in *a priori* defined anatomical ROIs for high calorie food against neutral non-food images

Kisspeptin	Vehicle		t	df	p
ACC (HC-N)	ACC (HC-N)	t-test	-0.062	26	0.951
		TOST	2.54	26	0.009
Accumbens (HC-N)	Accumbens (HC-N)	t-test	0.073	26	0.943
		TOST	-2.53	26	0.009
Amygdala (HC-N)	Amygdala (HC-N)	t-test	-0.220	26	0.828
		TOST	2.38	26	0.013
Caudate (HC-N)	Caudate (HC-N)	t-test	-0.547	26	0.589
		TOST	2.05	26	0.025
Globus Pallidus (HC-N)	Globus Pallidus (HC-N)	t-test	0.022	26	0.982
		TOST	-2.58	26	0.008
Hippocampus (HC-N)	Hippocampus (HC-N)	t-test	-0.577	26	0.569
		TOST	2.02	26	0.027
Insula (HC-N)	Insula (HC-N)	t-test	-0.045	26	0.964
		TOST	2.55	26	0.008
OFC (HC-N)	OFC (HC-N)	t-test	-0.287	26	0.776
		TOST	2.31	26	0.015
Putamen (HC-N)	Putamen (HC-N)	t-test	0.450	26	0.656
		TOST	-2.15	26	0.021
Thalamus (HC-N)	Thalamus (HC-N)	t-test	-0.212	26	0.834
		TOST	2.39	26	0.012
“Food reward” mask (HC-N)	“Food reward” mask (HC-N)	t-test	-0.185	26	0.855
		TOST	2.41	26	0.012

HC-N = high calorie food against neutral non-food images. Data presented as paired two-tailed t-tests and TOST (two one-sided t-tests) with upper and lower equivalence bounds set as Cohen’s d -0.5 and 0.5. TOST generates p values for the upper equivalence bound and the lower equivalence bound, the less significant p value is presented in the table above.

Table 4.8: Effects of kisspeptin compared to vehicle in *a priori* defined anatomical ROIs for low calorie food against neutral non-food images

Kisspeptin	Vehicle		t	df	p
ACC (LC-N)	ACC (LC-N)	t-test	0.483	26	0.633
		TOST	-2.12	26	0.022
Accumbens (LC-N)	Accumbens (LC-N)	t-test	0.626	26	0.537
		TOST	-1.97	26	0.030
Amygdala (LC-N)	Amygdala (LC-N)	t-test	-0.526	26	0.603
		TOST	2.07	26	0.024
Caudate (LC-N)	Caudate (LC-N)	t-test	-0.111	26	0.913
		TOST	2.49	26	0.010
Globus Pallidus (LC-N)	Globus Pallidus (LC-N)	t-test	0.321	26	0.751
		TOST	-2.28	26	0.016
Hippocampus (LC-N)	Hippocampus (LC-N)	t-test	-0.002	26	0.999
		TOST	-2.60	26	0.008
Insula (LC-N)	Insula (LC-N)	t-test	0.022	26	0.982
		TOST	-2.58	26	0.008
OFC (LC-N)	OFC (LC-N)	t-test	0.646	26	0.524
		TOST	-1.95	26	0.031
Putamen (LC-N)	Putamen (LC-N)	t-test	0.271	26	0.789
		TOST	-2.33	26	0.014
Thalamus (LC-N)	Thalamus (LC-N)	t-test	0.028	26	0.978
		TOST	-2.57	26	0.008
“Food reward” mask (LC-N)	“Food reward” mask (LC-N)	t-test	0.003	26	0.979
		TOST	-2.57	26	0.008

LC-N = low calorie food against neutral non-food images. Data presented as paired two-tailed t-tests and TOST (two one-sided t-tests) with upper and lower equivalence bounds set as Cohen’s d -0.5 and 0.5. TOST generates p values for the upper equivalence bound and the lower equivalence bound, the less significant p value is presented in the table above.

Table 4.9: Effects of kisspeptin compared to vehicle in *a priori* defined anatomical ROIs for high calorie food against low calorie food (subtracting neutral non-food images)

Kisspeptin	Vehicle		t	df	p
ACC (HC/N-LC/N)	ACC (HC/N-LC/N)	t-test	-0.647	26	0.524
		TOST	1.95	26	0.031
Accumbens (HC/N-LC/N)	Accumbens (HC/N-LC/N)	t-test	-0.619	26	0.541
		TOST	1.98	26	0.029
Amygdala (HC/N-LC/N)	Amygdala (HC/N-LC/N)	t-test	0.344	26	0.733
		TOST	-2.25	26	0.016
Caudate (HC/N-LC/N)	Caudate (HC/N-LC/N)	t-test	-0.708	26	0.485
		TOST	1.89	26	0.035
Globus Pallidus (HC/N-LC/N)	Globus Pallidus (HC/N-LC/N)	t-test	-0.371	26	0.714
		TOST	2.23	26	0.017
Hippocampus (HC/N-LC/N)	Hippocampus (HC/N-LC/N)	t-test	-0.605	26	0.551
		TOST	1.99	26	0.028
Insula (HC/N-LC/N)	Insula (HC/N-LC/N)	t-test	-0.089	26	0.930
		TOST	2.51	26	0.009
OFC (HC/N-LC/N)	OFC (HC/N-LC/N)	t-test	-1.265	26	0.217
		TOST	-3.86	26	0.001
Putamen (HC/N-LC/N)	Putamen (HC/N-LC/N)	t-test	0.329	26	0.745
		TOST	-2.27	26	0.016
Thalamus (HC/N-LC/N)	Thalamus (HC/N-LC/N)	t-test	-0.273	26	0.787
		TOST	2.33	26	0.014
"Food reward" mask (HC/N-LC/N)	"Food reward" mask (HC/N-LC/N)	t-test	-0.313	26	0.757
		TOST	2.29	26	0.015

HC/N-LC/N = high calorie food against low calorie food (subtracting neutral non-food images). Data presented as paired two-tailed t-tests and TOST (two one-sided t-tests) with upper and lower equivalence bounds set as Cohen's d -0.5 and 0.5. TOST generates p values for the upper equivalence bound and the lower equivalence bound, the less significant p value is presented in the table above.

4.3 Discussion

This is the first study to investigate the effects of peripheral kisspeptin administration on brain responses to food images in humans. In this cohort of healthy men, peripheral kisspeptin administered as a 1 nmol/kg/hour infusion did not have any effects on brain processing in response to food images at the whole brain and ROI level. Comparable with my findings in Chapter 3, kisspeptin administration also had no significant effects on appetite parameters as assessed by FCQ and VAS questions.

In this study, an intravenous kisspeptin-54 infusion was administered at a dose known to be bioactive in healthy men as demonstrated by increase in serum LH without downstream rise in testosterone levels, which is in agreement with my findings in Chapters 2 - 3 and in keeping with previous work (Comninou *et al.*, 2017). Furthermore, peripherally administered kisspeptin-54 is capable of penetrating the blood-brain barrier to reach deeper brain structures known to express kisspeptin and its receptor (d'Anglemont de Tassigny *et al.*, 2017; Comninou *et al.*, 2017). Thus, methodologically, the route and dose of administration of kisspeptin used in this study was sufficient to access brain regions involved in food-reward and appetite in humans.

Efforts were also made to reduce sources of variability, and control for potential confounders by recruiting healthy men of similar age with no abnormalities in baseline eating behaviours. All participants followed the same instructions regarding food, alcohol and caffeine intake prior to each study visit. Additionally, study visits were performed in a randomised order with participants blinded to the contents of intravenous infusions.

Previous work demonstrates that peripheral kisspeptin administration enhances brain reward pathways in response to sexual and couple-bonding images (Comninou *et al.*, 2017). Subsequently, my findings in Chapter 2 reveal that peripheral kisspeptin administration also enhances hedonic brain pathways in response to a feminine olfactory stimulus. As food

images, which are considered another form of rewarding stimuli, did not elicit a significant kisspeptin-enhanced brain response in this study, this suggests that kisspeptin's effects on limbic brain pathways are specific to sexual and attraction stimuli. Furthermore, when considering the lack of kisspeptin-effect on brain regions known to be associated with reward and appetite-regulating pathways, TOST equivalence testing confirmed that the results were most likely due to a true null effect, rather than low experimental power.

Taken together, the neuroimaging and psychometric data presented in this chapter provides further support for my findings in Chapter 3 that peripheral kisspeptin administration does not acutely affect appetite or *ad libitum* food intake in healthy men. Collectively, the results from Chapters 3 and 4 confirm that peripheral administration of kisspeptin-54 given at 1 nmol/kg/hour to healthy men does not alter brain responses to food stimuli, psychometric parameters of appetite nor *ad libitum* food intake in the acute setting.

4.3.1 Limitations

Consistent with previous chapters, this was another novel proof-of-concept study thus to reduce variability, participants were all healthy eugonadal young men with a narrow BMI range. Therefore, these findings may not be generalisable to other populations including women, and patients with metabolic disorders such as diabetes mellitus. Female *Kiss1r* knockout mice display different bodyweight changes and metabolic derangements compared to *Kiss1r* knockout male mice (Tolson *et al.*, 2014; Velasco *et al.*, 2019) and hypothalamic *Kiss1* expression is reduced in diabetic rodents with normalisation of LH and testosterone following kisspeptin administration (Castellano *et al.*, 2006; Dudek *et al.*, 2016). Therefore, it is important to carry out further human studies to assess for differences in kisspeptin's effects on appetite and food intake in these groups. If indeed there is a difference in food-behaviour in response to kisspeptin, then fMRI would be a valuable modality for investigating the brain mechanisms underlying these observations.

4.3.2 Summary

Peripheral administration of kisspeptin-54 at a dose of 1 nmol/kg/hour (which robustly increases LH) does not affect psychometric parameters of appetite nor brain response to food images as measured by fMRI BOLD activity. This is the first human fMRI study to investigate the effects of kisspeptin on brain pathways regulating appetite and food-reward behaviours. These findings support my behavioural and food intake data in Chapter 3 and contribute to our understanding of kisspeptin's roles within reproductive and metabolic pathways in humans.

4.3.3 Future work

As discussed in the limitations above, this is a small cohort study of healthy young men with narrow BMI range and further studies are required to explore the effects of kisspeptin in different groups such as women and patients with hypogonadism or diabetes mellitus. In this study, exogenous kisspeptin was administered, which may have differing effects compared to endogenous kisspeptin release in the brain, thus the physiological role of kisspeptin within human brain pathways regulating food-reward and appetite remains unexplored. One potential way to assess endogenous kisspeptin signalling in these brain pathways would be to perform positron emission tomography using a radiolabelled kisspeptin tracer to visualise endogenous kisspeptin uptake under different experimental conditions. However, no such tracer currently exists and the estimated cost of manufacturing such a novel tracer and carrying out these studies is prohibitive. An alternative method would be the use of kisspeptin receptor antagonists (Roseweir *et al.*, 2009) to assess the effects on brain pathways when endogenous kisspeptin signalling is blocked. However, the effects of global kisspeptin receptor blockade on the reproductive axis and other downstream neurotransmitter pathways known to interact with kisspeptin would be significant confounders in these studies.

Chapter 5

General Discussion

5.1 General discussion

Since the discovery of the *KISS1* gene in 1996 (Lee *et al.*, 1996), the kisspeptin family of neuropeptides have become established as key regulators of the reproductive axis (de Roux *et al.*, 2003; Seminara *et al.*, 2003) with studies demonstrating their potential as novel clinical treatments for reproductive disorders (Jayasena *et al.*, 2014a, 2014b; Abbara *et al.*, 2015). The expression of kisspeptin and its receptor has also been observed in multiple brain regions outside the classical hypothalamic reproductive circuit in rodents and humans (Lee *et al.*, 1999; Kotani *et al.*, 2001; Muir *et al.*, 2001; Gottsch *et al.*, 2004; Clarkson *et al.*, 2009; Herbison *et al.*, 2010). More recently, studies investigating the role of kisspeptin signalling in these extra-hypothalamic brain tissues have revealed wide-reaching effects on sexual behaviours as well as olfaction, mood and emotions in animals (Kauffman *et al.*, 2007; Pineda *et al.*, 2017; Adekunbi *et al.*, 2018; Aggarwal *et al.*, 2018; Hellier *et al.*, 2018). This expanding body of work demonstrates a complex network of afferent and efferent connections as well as excitatory and inhibitory signals providing a functional and neuroanatomical framework through which kisspeptin-signalling may integrate multiple internal and external cues with appropriate behavioural and neuroendocrine responses (Comninou & Dhillo, 2017).

Prior to this thesis, only one human study had been conducted to investigate the effects of kisspeptin on extra-hypothalamic brain pathways. The findings showed that peripheral kisspeptin administration enhanced limbic brain processing in response to sexually arousing and couple-bonding images in healthy men, and was associated with reward measures and reduced sexual aversion (Comninou *et al.*, 2017). Following on from this, several outstanding areas of investigation remain with regard to interrogating the effects of kisspeptin on human brain pathways involved in processing other external cues e.g. olfactory stimuli, opposite-sex attraction and metabolic signals. Furthermore, kisspeptin has been implicated as an integral link between metabolic signals and the reproductive axis (Tena-Sempere, 2017; Navarro, 2020). However, until now *in vivo* human studies have not been performed to investigate the effects of kisspeptin on human appetite and feeding behaviour.

The aims of this PhD thesis were to explore the effects of kisspeptin on two distinct aspects of human behaviour that have not yet been addressed:-

1. Brain responses to olfactory and visual cues of attraction and the pathways that underlie these behaviours.
2. Change in appetite and *ad libitum* food intake, along with associated brain pathways regulating food-reward behaviour.

To achieve this, I conducted three separate human studies detailed in Chapters 2 to 4, with my main findings summarised as follows:

Chapter 2: Kisspeptin enhances brain responses to olfactory and visual cues of attraction in healthy men

This study utilised fMRI, psychometric and hormone analyses to demonstrate that peripheral kisspeptin administration enhances brain responses to olfactory and visual cues of attraction, with augmented kisspeptin effects in men with lower behavioural reward drive and sexual quality of life. As such, this reveals a previously undescribed kisspeptin-activated attraction pathway, uniting two major sensory inputs of human attraction with corresponding neural processing and reproductive hormonal control. These results add to the literature surrounding kisspeptin's effects on human psychosexual function and propose a new and exciting avenue for manipulation of kisspeptin-mediated pathways to deliver clinical strategies for related reproductive and psychosexual disorders.

Chapter 3: Kisspeptin does not affect appetite or *ad libitum* food intake in healthy men

This was a proof-of-concept study in healthy men to investigate the effects of peripheral kisspeptin administration on appetite parameters and food intake during an *ad libitum* meal. The results reveal that kisspeptin has no significant effects on appetite parameters in the fasted or fed state, with no differences in *ad libitum* food intake.

Chapter 4: Kisspeptin does not affect brain responses to food imagery in healthy men

This study was performed in tandem with the study in Chapter 3 and utilised fMRI, psychometric and hormone analyses to investigate the effects of peripheral kisspeptin administration on human brain pathways regulating appetite and food-reward. The results showed no significant effects of kisspeptin on brain responses to viewing food images, which provides mechanistic support for the behavioural observations in Chapter 3.

The human data presented here successfully supports and extends the animal literature, which has elicited strong roles for kisspeptin in olfactory-mediated sexual attraction and various sexual behaviours (Kauffman *et al.*, 2007; Gresham *et al.*, 2016; Aggarwal *et al.*, 2018; Adekunbi *et al.*, 2018; Hellier *et al.*, 2018). On the other hand, the animal data surrounding kisspeptin's function within the regulation of appetite is more conflicted, with an incomplete understanding of the interactions between kisspeptin and appetite-regulating pathways (Fu & Van Den Pol, 2010; Backholer *et al.*, 2010; Talbi *et al.*, 2016; Cázarez-Márquez *et al.*, 2019) nor whether kisspeptin administration definitively affects food intake (Thompson *et al.*, 2004; Castellano *et al.*, 2005; Saito *et al.*, 2019). Moreover, very little is known about the role of extra-hypothalamic kisspeptin populations in appetite-regulating pathways and food intake in any species, and my study in Chapter 4 is the first to provide mechanistic insight into the effects of kisspeptin on human brain pathways related to appetite and food-reward behaviours.

Collectively, the findings of this PhD thesis generate novel human data that furthers our understanding of human psychosexual function, appetite and food intake by demonstrating that kisspeptin administration significantly enhances brain pathways in response to olfactory and visual cues of attraction, but does not have a significant effect on appetite-regulating brain pathways nor on food intake in healthy men.

5.2 Limitations and future work

A potential limitation that transcends all three studies is that the participants were healthy eugonadal young men with a narrow BMI range and therefore these results may not be generalisable to other populations. Much is still unknown with regard to human psychosexual function, and in this thesis I have only examined attraction in heterosexual men, thus future work to include men and women with different sexual orientations would be useful to elicit any contrasting effects of kisspeptin on these brain processes.

In Chapter 2, a synthetic olfactory stimulus (Chanel No.5) was chosen as a robust feminine scent based on previously published fMRI data (Huh *et al.*, 2008), however sources of endogenous opposite-sex odours e.g. clothing worn by women in various phases of the menstrual cycle (Miller & Maner, 2010) would be an alternative stimulus that is worth exploring in future.

I observed that peripheral kisspeptin administration was able to enhance brain regions related to olfactory processing, reward and sexual arousal. These regions match areas where human kisspeptin receptor is expressed (Muir *et al.*, 2001; Kotani *et al.*, 2001), suggesting a direct receptor-mediated action of kisspeptin here. However, it is also important to consider additional pathways that may be involved in exerting the observed downstream effects of kisspeptin on the brain. Kisspeptin activates GnRH neurons which are also distributed in numerous extra-hypothalamic tissues (Kubek *et al.*, 1979; Rance *et al.*, 1994; Stopa *et al.*, 1991). However, in this study kisspeptin also enhanced brain activity in areas that are not known to contain GnRH neurons or GnRH receptors in humans (including the caudate, globus pallidus, insula and PCC) (Kubek *et al.*, 1979; Rance *et al.*, 1994; Stopa *et al.*, 1991; Wilson *et al.*, 2006), which suggests there are GnRH-independent actions of kisspeptin in these regions. Furthermore, there is functional evidence indicating that kisspeptin-induced sexual behaviours including lumbar lordosis in mice and ex-copula erections in rats can occur independently of GnRH (Hellier *et al.*, 2018; Gresham *et al.*, 2016). Additionally, kisspeptin

has been shown to interact with numerous other neuropeptide systems including serotonin (Nathan *et al.*, 2015a; Tanaka *et al.*, 2013), dopamine (Pineda *et al.*, 2017), vasopressin (Pineda *et al.*, 2017), gamma-aminobutyric acid (Di Giorgio *et al.*, 2014), glutamate (Nathan *et al.*, 2015b) and nitric oxide (Hanchate *et al.*, 2012; Tena-Sempere, 2017; Hellier *et al.*, 2018). Hence, further work is necessary to unravel potential interactions between the kisspeptin system and other neurotransmitters that may be responsible for orchestrating the kisspeptin-mediated changes in brain responses seen in this thesis and underlying the effects of kisspeptin on sexual and emotional brain pathways demonstrated in previous work (Comninou *et al.*, 2017).

Another consideration is that despite enhancement of brain responses to olfactory and visual cues of attraction, no differences in participants' ratings of the olfactory and facial stimuli were detected between kisspeptin and vehicle visits. As discussed previously, the assessment of these ratings was limited to 20-point and 5-point scales for olfactory and facial stimuli respectively, which may not be sensitive enough to detect small changes in perception of attraction. Furthermore, the studies in Chapters 3 and 4 also did not detect any significant changes in psychometric parameters of appetite during kisspeptin administration compared to vehicle. However, it is worth noting that none of the studies in this thesis were powered to detect changes in psychometric parameters, as the primary outcomes were change in fMRI BOLD activity (Chapters 2 and 4) and change in food intake (Chapter 3).

In terms of kisspeptin's effects on appetite-pathways and food intake, an important next step would be to perform human studies in women, as female *Kiss1r* knockout mice display different bodyweight changes and metabolic derangements compared to *Kiss1r* knockout male mice (Tolson *et al.*, 2014; Velasco *et al.*, 2019). It would also be useful to explore the metabolic effects of kisspeptin in patients with hypogonadism as this condition can be associated with derangements in nutritional intake and energy balance (Couzinet *et al.*, 1999). Moreover, hypothalamic *Kiss1* expression is reduced in rodent models of diabetes with

normalisation of LH and testosterone following kisspeptin administration (Castellano *et al.*, 2006, 2009; Dudek *et al.*, 2016). Similarly, kisspeptin administration to diabetic hypogonadal men is also able to stimulate LH and testosterone (George *et al.*, 2013). In this thesis, I have focused on investigating the effects of kisspeptin within central appetite-regulating processes and shown no significant changes. There is also evidence indicating that kisspeptin has effects on other aspects of metabolism as demonstrated by deranged glucose tolerance in *Kiss1r* knockout female mice (Tolson *et al.*, 2014), and to a lesser extent in high-fat fed *Kiss1r* knockout male mice (Velasco *et al.*, 2019). Furthermore, kisspeptin may have an emerging role in beta cell biology as kisspeptin administration has been shown to affect glucose-stimulated insulin release in rodents and humans, albeit with some conflicting results between *in vitro* and *in vivo* studies (Hauge-Evans *et al.*, 2006; Chen *et al.*, 2014; Izzi-Engbeaya *et al.*, 2018). Taken together, diabetic populations would be a very useful cohort in which to investigate the effects of kisspeptin on metabolic parameters such as appetite, food intake, and beta-cell biology.

Another potential limitation and area of future work is that all the studies in this thesis have involved the administration of exogenous kisspeptin, achieving plasma kisspeptin levels similar to physiological levels observed in normal pregnancy (Horikoshi *et al.*, 2003) and comparable to levels required to restore reproductive function in hypothalamic amenorrhoea (Jayasena *et al.*, 2014b). Although this supports the robustness of my findings that a bioactive dose of kisspeptin can activate attraction-related brain pathways but does not affect food-related brain processing or food intake, this may not represent the effects of endogenous kisspeptin release in the brain. Potential ways to explore endogenous kisspeptin signalling in human brain pathways could involve the use of other functional neuroimaging techniques such as positron emission tomography using a radiolabelled kisspeptin tracer. However, such a tracer is not currently available, and the high financial cost of developing one and performing these studies would be a limiting factor. An alternative approach to interrogate endogenous kisspeptin signalling in humans would be the use of kisspeptin receptor antagonists (Roseweir

et al., 2009). However, the effects of these agents require further assessment in humans to examine their blood-brain barrier penetrance and consider the consequences of global kisspeptin receptor blockade on the reproductive axis and other downstream neurotransmitter pathways known to interact with kisspeptin, which could significantly confound studies involving these compounds.

5.3 Summary

Overall, this PhD thesis provides novel evidence for a specific effect of kisspeptin on brain responses to olfactory and visual attraction cues but not food cues. This broadens our understanding of kisspeptin's role in human psychosexual function and provides further evidence for the development of kisspeptin-based therapies to treat related reproductive and psychosexual disorders. Determining a lack of effect of kisspeptin on brain pathways regulating appetite and food-reward behaviours with no changes in appetite parameters and food intake answers key questions relating to the role of kisspeptin in human appetite.

Currently there remains a gap in our knowledge between the effects of kisspeptin in animal models and humans that needs to be bridged by conducting further studies to explore kisspeptin's effects on other aspects of human emotion and behaviour e.g. in patients with psychosexual dysfunction and/or mood disorders. The fMRI paradigm used in this thesis has proven to be an excellent tool for exploring the mechanistic effects of kisspeptin on brain pathways of interest, so future work incorporating fMRI, psychometric and behavioural outputs would be an indispensable framework for assessing the effects of kisspeptin.

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Appendix 1: MRI Safety Screening Questionnaire

Questions	YES (Initial)	NO (initial)
Do you suffer with claustrophobia? (fear of small/cramped spaces)		
Do you have a heart pacemaker or pacing wires?		
Have you ever had any operations on your heart or heart implants?		
Have you ever had any operations on your head or brain?		
Have you ever had eye surgery or implants?		
Have you ever worked with metal i.e. grinding/machining/welding?		
Have you EVER had an injury to or around the eyes involving metal i.e. metal fragments/swarf?		
Have you ever had a metallic injury to any other part of your body?		
Do you wear a hearing aid, or have an internal ear implant e.g. cochlear implant?		
Do you have a denture plate/bridge or other metallic dental work other than fillings?		
Do you have any transdermal (skin) patches?		
Do you have any implanted devices e.g. IUD, nerve stimulator, pump, programmable brain shunt.		
Do you have any artificial limb(s) body prosthesis or support of any kind?		
Do you have any metallic body piercing, tattoos/tattooed eyeliner/hair extensions?		
Have you ever had an operation involving metallic plates/pins/screws or wires?		
Have you had an operation in the last 3 months?		
Have you ever been involved in a serious accident e.g. road traffic accident/explosive injury/shooting injury?		
Have you ever had any other surgical procedure of any kind?		

Investigator's comments:

Appendix 2: Trait questionnaires used in Chapter 2

BIS/BAS Questionnaire – Page 1 /2

For each item, indicate how much you agree or disagree with what the item says. Please respond to all the items. Choose only one response to each statement. Please be as accurate and honest as you can be. Respond to each item as if it were the only item. That is, don't worry about being "consistent" in your responses. Choose from the following four response options:

1 = very true for me

2 = somewhat true for me

3 = somewhat false for me

4 = very false for me

1. A person's family is the most important thing in life. _____
2. Even if something bad is about to happen to me, I rarely experience fear or nervousness. _____
3. I go out of my way to get things I want. _____
4. When I'm doing well at something I love to keep at it. _____
5. I'm always willing to try something new if I think it will be fun. _____
6. How I dress is important to me. _____
7. When I get something I want, I feel excited and energized. _____
8. Criticism or scolding hurts me quite a bit. _____
9. When I want something I usually go all-out to get it. _____
10. I will often do things for no other reason than that they might be fun. _____
11. It's hard for me to find the time to do things such as get a haircut. _____
12. If I see a chance to get something I want I move on it right away. _____
13. I feel pretty worried or upset when I think or know somebody is angry at me. _____

BIS/BAS Questionnaire – Page 2 / 2

For each item, indicate how much you agree or disagree with what the item says. Please respond to all the items. Choose only one response to each statement. Please be as accurate and honest as you can be. Respond to each item as if it were the only item. That is, don't worry about being "consistent" in your responses. Choose from the following four response options:

- 1 = very true for me**
- 2 = somewhat true for me**
- 3 = somewhat false for me**
- 4 = very false for me**

- 14. When I see an opportunity for something I like I get excited right away. _____
- 15. I often act on the spur of the moment. _____
- 16. If I think something unpleasant is going to happen I usually get pretty "worked up." _____
- 17. I often wonder why people act the way they do. _____
- 18. When good things happen to me, it affects me strongly. _____
- 19. I feel worried when I think I have done poorly at something important. _____
- 20. I crave excitement and new sensations. _____
- 21. When I go after something I use a "no holds barred" approach. _____
- 22. I have very few fears compared to my friends. _____
- 23. It would excite me to win a contest. _____
- 24. I worry about making mistakes. _____

INTERNATIONAL INDEX OF ERECTILE FUNCTION

Please try to answer the questions as honestly and as clearly as you are able. In answering the questions, the following definitions apply:

- **sexual activity** includes intercourse, caressing, foreplay & masturbation
- **sexual intercourse** is defined as sexual penetration of your partner
- **sexual stimulation** includes situation such as foreplay, erotic pictures etc.
- **ejaculation** is the ejection of semen from the penis (or the feeling of this)
- **orgasm** is the fulfilment or climax following sexual stimulation or intercourse

OVER THE PAST 4 WEEKS
CHECK ONE BOX ONLY

☐ Q1	How often were you able to get an erection during sexual activity?	0 No sexual activity 1 Almost never or never 2 A few times (less than half the time) 3 Sometimes (about half the time) 4 Most times (more than half the time) 5 Almost always or always
☐ Q2	When you had erections with sexual stimulation, how often were your erections hard enough for penetration?	0 No sexual activity 1 Almost never or never 2 A few times (less than half the time) 3 Sometimes (about half the time) 4 Most times (more than half the time) 5 Almost always or always
☐ Q3	When you attempted intercourse, how often were you able to penetrate (enter) your partner?	0 Did not attempt intercourse 1 Almost never or never 2 A few times (less than half the time) 3 Sometimes (about half the time) 4 Most times (more than half the time) 5 Almost always or always
☐ Q4	During sexual intercourse, <u>how often</u> were you able to maintain your erection after you had penetrated (entered) your partner?	0 Did not attempt intercourse 1 Almost never or never 2 A few times (less than half the time) 3 Sometimes (about half the time) 4 Most times (more than half the time) 5 Almost always or always
☐ Q5	During sexual intercourse, <u>how difficult</u> was it to maintain your erection to completion of intercourse?	0 Did not attempt intercourse 1 Extremely difficult 2 Very difficult 3 Difficult 4 Slightly difficult 5 Not difficult

☐ Q6	How many times have you attempted sexual intercourse?	0 No attempts 1 One to two attempts 2 Three to four attempts 3 Five to six attempts 4 Seven to ten attempts 5 Eleven or more attempts
☐ Q7	When you attempted sexual intercourse, how often was it satisfactory for you?	0 Did not attempt intercourse 1 Almost never or never 2 A few times (less than half the time) 3 Sometimes (about half the time) 4 Most times (more than half the time) 5 Almost always or always
☐ Q8	How much have you enjoyed sexual intercourse?	0 No intercourse 1 No enjoyment at all 2 Not very enjoyable 3 Fairly enjoyable 4 Highly enjoyable 5 Very highly enjoyable
☐ Q9	When you had sexual stimulation <u>or</u> intercourse, how often did you ejaculate?	0 No sexual stimulation or intercourse 1 Almost never or never 2 A few times (less than half the time) 3 Sometimes (about half the time) 4 Most times (more than half the time) 5 Almost always or always
☐ Q10	When you had sexual stimulation <u>or</u> intercourse, how often did you have the feeling of orgasm or climax?	1 Almost never or never 2 A few times (less than half the time) 3 Sometimes (about half the time) 4 Most times (more than half the time) 5 Almost always or always
☐ Q11	How often have you felt sexual desire?	1 Almost never or never 2 A few times (less than half the time) 3 Sometimes (about half the time) 4 Most times (more than half the time) 5 Almost always or always
☐ Q12	How would you rate your level of sexual desire?	1 Very low or none at all 2 Low 3 Moderate 4 High 5 Very high
☐ Q13	How satisfied have you been with your <u>overall sex life</u> ?	1 Very dissatisfied 2 Moderately dissatisfied 3 Equally satisfied & dissatisfied 4 Moderately satisfied 5 Very satisfied
☐ Q14	How satisfied have you been with your <u>sexual relationship</u> with your partner?	1 Very dissatisfied 2 Moderately dissatisfied 3 Equally satisfied & dissatisfied 4 Moderately satisfied 5 Very satisfied
☐ Q15	How do you rate your <u>confidence</u> that you could get and keep an erection?	1 Very low 2 Low 3 Moderate 4 High 5 Very high

Sexual Desire Inventory-2 (SDI-2)

This questionnaire asks about your level of sexual desire. By desire, we mean INTEREST IN or WISH FOR SEXUAL ACTIVITY. For each item, please circle the number that best shows your thoughts and feelings. Your answers will be private and anonymous.

1. During the last month, how often would you have liked to engage in sexual activity with a partner (for example, touching each other's genitals, giving or receiving oral stimulation, intercourse, etc.)?

0) Not at all	4) Twice a week
1) Once a month	5) 3 to 4 times a week
2) Once every two weeks	6) Once a day
3) Once a week	7) More than once a day

2. During the last month, how often have you had sexual thoughts involving a partner?

0) Not at all	4) 3 to 4 times a week
1) Once or twice a month	5) Once a day
2) Once a week	6) A couple of times a day
3) Twice a week	7) Many times a day

3. When you have sexual thoughts, how strong is your desire to engage in sexual behavior with a partner?

0	1	2	3	4	5	6	7	8
No Desire					Strong Desire			

4. When you first see an attractive person, how strong is your sexual desire?

0	1	2	3	4	5	6	7	8
No Desire					Strong Desire			

5. When you spend time with an attractive person (for example, at work or school), how strong is your sexual desire?

0	1	2	3	4	5	6	7	8
No Desire					Strong Desire			

6. When you are in romantic situations (such as a candle lit dinner, a walk on the beach, etc.), how strong is your sexual desire?

0	1	2	3	4	5	6	7	8
No Desire					Strong Desire			

7. How strong is your desire to engage in sexual activity with a partner?

0 1 2 3 4 5 6 7 8
No Desire Strong Desire

8. How important is it for you to fulfill your sexual desire through activity with a partner?

0 1 2 3 4 5 6 7 8
Not At All Extremely
Important Important

9. Compared to other people of your age and sex, how would you rate your desire to behave sexually with a partner?

0 1 2 3 4 5 6 7 8
Much Less Much More
Desire Desire

10. During the last month, how often would you have liked to behave sexually by yourself (for example, masturbating, touching your genitals etc.)?

0) Not at all 4) Twice a week
1) Once a month 5) 3 to 4 times a week
2) Once every two weeks 6) Once a day
3) Once a week 7) More than once a day

11. How strong is your desire to engage in sexual behavior by yourself?

0 1 2 3 4 5 6 7 8
No Desire Strong Desire

12. How important is it for you to fulfill your desires to behave sexually by yourself?

0 1 2 3 4 5 6 7 8
Not At All Extremely
Important Important

13. Compared to other people of your age and sex, how would you rate your desire to behave sexually by yourself?

0 1 2 3 4 5 6 7 8
Much Less Much More
Desire Desire

14. How long could you go comfortably without having sexual activity of some kind?

0) Forever 5) A week
1) A year or two 6) A few days
2) Several months 7) One day
3) A month 8) Less than one day
4) A few weeks

SELF-EVALUATION QUESTIONNAIRE

STAI Form Y-2

DIRECTIONS

A number of statements which people have used to describe themselves are given below. Read each statement and then circle the appropriate number to the right of the statement to indicate how you *generally* feel. There are no right or wrong answers. Do not spend too much time on any one statement but give the answer which seems to describe how you generally feel.

ALMOST NEVER
SOMETIMES
OFTEN
ALMOST ALWAYS

- | | 1 | 2 | 3 | 4 |
|--|---|---|---|---|
| 21. I feel pleasant..... | 1 | 2 | 3 | 4 |
| 22. I feel nervous and restless | 1 | 2 | 3 | 4 |
| 23. I feel satisfied with myself..... | 1 | 2 | 3 | 4 |
| 24. I wish I could be as happy as others seem to be | 1 | 2 | 3 | 4 |
| 25. I feel like a failure | 1 | 2 | 3 | 4 |
| 26. I feel rested | 1 | 2 | 3 | 4 |
| 27. I am "calm, cool, and collected" | 1 | 2 | 3 | 4 |
| 28. I feel that difficulties are piling up so that I cannot overcome them..... | 1 | 2 | 3 | 4 |
| 29. I worry too much over something that really doesn't matter..... | 1 | 2 | 3 | 4 |
| 30. I am happy | 1 | 2 | 3 | 4 |
| 31. I have disturbing thoughts | 1 | 2 | 3 | 4 |
| 32. I lack self-confidence..... | 1 | 2 | 3 | 4 |
| 33. I feel secure | 1 | 2 | 3 | 4 |
| 34. I make decisions easily | 1 | 2 | 3 | 4 |
| 35. I feel inadequate..... | 1 | 2 | 3 | 4 |
| 36. I am content | 1 | 2 | 3 | 4 |
| 37. Some unimportant thought runs through my mind and bothers me | 1 | 2 | 3 | 4 |
| 38. I take disappointments so keenly that I can't put them out of my mind | 1 | 2 | 3 | 4 |
| 39. I am a steady person..... | 1 | 2 | 3 | 4 |
| 40. I get in a state of tension or turmoil as I think over my recent concerns
and interests | 1 | 2 | 3 | 4 |

Sexual Quality of Life – Male

This questionnaire consists of a set of statements, each asking about thoughts and feelings that you may have about your sexual life. The statement may be positive or negative.

You are asked to rate each one according to how much you agree or disagree with the statement by a circling one of six categories.

In answering these items the following definitions apply:

Sexual life: is both the physical sexual activities and the emotional sexual relationship that you have with your partner.

Sexual activity: Includes any activity which may result in sexual stimulation or sexual pleasure e.g. intercourse, caressing, foreplay, masturbation (i.e. self masturbation or your partner masturbating you) and oral sex (i.e. your partner giving you oral sex).

Usually, the first answer that comes into your head is the best one so, do not spend too long on each question.

All your answers will be completely confidential

	1	2	3	4	5	6
1. When I think about my sexual life, I feel frustrated	<i>completely agree</i>	<i>moderately agree</i>	<i>slightly agree</i>	<i>slightly disagree</i>	<i>moderately disagree</i>	<i>completely disagree</i>
2. When I think about my sexual life, I feel depressed	<i>completely agree</i>	<i>moderately agree</i>	<i>slightly agree</i>	<i>slightly disagree</i>	<i>moderately disagree</i>	<i>completely disagree</i>
3. When I think about my sexual life, I feel like less of a man	<i>completely agree</i>	<i>moderately agree</i>	<i>slightly agree</i>	<i>slightly disagree</i>	<i>moderately disagree</i>	<i>completely disagree</i>
4. I have lost confidence in myself as a sexual partner	<i>completely agree</i>	<i>moderately agree</i>	<i>slightly agree</i>	<i>slightly disagree</i>	<i>moderately disagree</i>	<i>completely disagree</i>
5. When I think about my sexual life, I feel anxious	<i>completely agree</i>	<i>moderately agree</i>	<i>slightly agree</i>	<i>slightly disagree</i>	<i>moderately disagree</i>	<i>completely disagree</i>
6. When I think about my sexual life, I feel angry	<i>completely agree</i>	<i>moderately agree</i>	<i>slightly agree</i>	<i>slightly disagree</i>	<i>moderately disagree</i>	<i>completely disagree</i>
7. I worry about the future of my sexual life	<i>completely agree</i>	<i>moderately agree</i>	<i>slightly agree</i>	<i>slightly disagree</i>	<i>moderately disagree</i>	<i>completely disagree</i>
8. When I think about my sexual life, I am embarrassed	<i>completely agree</i>	<i>moderately agree</i>	<i>slightly agree</i>	<i>slightly disagree</i>	<i>moderately disagree</i>	<i>completely disagree</i>
9. When I think about my sexual life, I feel guilty	<i>completely agree</i>	<i>moderately agree</i>	<i>slightly agree</i>	<i>slightly disagree</i>	<i>moderately disagree</i>	<i>completely disagree</i>
10. When I think about my sexual life, I worry that my partner feels hurt or rejected	<i>completely agree</i>	<i>moderately agree</i>	<i>slightly agree</i>	<i>slightly disagree</i>	<i>moderately disagree</i>	<i>completely disagree</i>
11. When I think about my sexual life, I feel like I have lost something	<i>completely agree</i>	<i>moderately agree</i>	<i>slightly agree</i>	<i>slightly disagree</i>	<i>moderately disagree</i>	<i>completely disagree</i>

Subjective Happiness Scale

Instructions: For each of the following statements and/or questions, please circle the point on the scale that you feel is most appropriate in describing you.

1. In general, I consider myself:

not a very happy person 1 2 3 4 5 6 7 a very happy person

2. Compared to most of my peers, I consider myself:

less happy 1 2 3 4 5 6 7 more happy

3. Some people are generally very happy. They enjoy life regardless of what is going on, getting the most out of everything. To what extent does this characterization describe you?

not at all 1 2 3 4 5 6 7 a great deal

4. Some people are generally not very happy. Although they are not depressed, they never seem as happy as they might be. To what extent does this characterization describe you?

not at all 1 2 3 4 5 6 7 a great deal

Satisfaction with Life Scale

Instructions: Below are five statements that you may agree or disagree with. Using the 1 - 7 scale below, indicate your agreement with each item by placing the appropriate number on the line preceding that item. Please be open and honest in your responding.

- 7 - Strongly agree
- 6 - Agree
- 5 - Slightly agree
- 4 - Neither agree nor disagree
- 3 - Slightly disagree
- 2 - Disagree
- 1 - Strongly disagree

_____ In most ways my life is close to my ideal.

_____ The conditions of my life are excellent.

_____ I am satisfied with my life.

_____ So far I have gotten the important things I want in life.

_____ If I could live my life over, I would change almost nothing.

Appendix 3: State questionnaires used in Chapter 2

The Sexual Arousal and Desire Inventory (SADI)

Sexual Arousal is defined as the **physiological responses that accompany or follow sexual desire**. For example, when you feel sexually aroused, your heart might beat faster or your palms may get sweaty. Men may experience penile erection, and women may feel a moistness of the vagina. Sexual Arousal involves the more physiological aspects of wanting sex. **Sexual desire is defined as an energizing force that motivates a person to seek out or initiate sexual contact and behaviour**. You can think of it as a hunger or a sexual 'drive' that leads you to seek out sexual contact. Sexual desire involves the more psychological aspects of wanting sex.

Keep in mind the definitions of sexual arousal and sexual desire. Following is a list of words that might **describe how you feel currently**. Different people experience sexual arousal and desire in distinct, individual ways. There is no 'right' or 'wrong' answer. Please indicate to what extent each word describes how you feel currently, by placing the number that describes the feeling most accurately.

Please use the following scale to rate each of the following words below. Please rate all the words. Do not skip any.

0	1	2	3	4	5
does not describe it at all			describes it moderately well		describes it perfectly
Anticipatory____			Frustrated____		
Tingly all over____			Lustful____		
Restrained____			Entranced____		
Anxious____			Aversion____		
Driven____			Hot____		
Frigid____			Tempted____		
Sensitive to touch____			Passionate____		
Sluggish____			Fantasize about sex____		
Urge to satisfy and/or be satisfied____			Repressed____		
Enthusiastic____			Disturbed____		
Unhappy____			Flushed____		
Wet (women only)____			Impatient____		
Hard (men only)____			Sensual____		
Resistant____			Breathe faster/pant____		
Warm all over____			Displeasure____		
Excited____			Stimulated____		
Tingling in genital area____			Tingling sensation in gut____		
Uninterested____			I forget about everything else____		
Pleasure____			Repulsion____		
Heart beats faster____			Sexy____		
Happy____			Quivering sensations____		
Angry____			Insensible____		
Attractive____			Seductive____		
Powerful____			Genitals reddish____		
Naughty____			Unattractive____		
Alluring____			Good____		
Lethargic____			Throbs in genital area____		
Horny____					

Profile of Mood States 2 Short Form for Adults (POMS2A)

The following is a list of words that describe feelings that people have. Please read each word carefully, then select the choice that best describes: how you feel **RIGHT NOW**.

Right now I feel.....

		Not at all	A little	Moderately	Quite a bit	Extremely
1	Friendly	☐	☐	☐	☐	☐
2	Tense	☐	☐	☐	☐	☐
3	Angry	☐	☐	☐	☐	☐
4	Worn out	☐	☐	☐	☐	☐
5	Lively	☐	☐	☐	☐	☐
6	Confused	☐	☐	☐	☐	☐
7	Considerate	☐	☐	☐	☐	☐
8	Sad	☐	☐	☐	☐	☐
9	Active	☐	☐	☐	☐	☐
10	Grouchy	☐	☐	☐	☐	☐
11	Energetic	☐	☐	☐	☐	☐
12	Panicky	☐	☐	☐	☐	☐
13	Hopeless	☐	☐	☐	☐	☐
14	Uneasy	☐	☐	☐	☐	☐
15	Unable to concentrate	☐	☐	☐	☐	☐
16	Fatigued	☐	☐	☐	☐	☐
17	Helpful	☐	☐	☐	☐	☐
18	Nervous	☐	☐	☐	☐	☐
19	Miserable	☐	☐	☐	☐	☐
20	Muddled	☐	☐	☐	☐	☐
21	Bitter	☐	☐	☐	☐	☐
22	Exhausted	☐	☐	☐	☐	☐
23	Anxious	☐	☐	☐	☐	☐
24	Good natured	☐	☐	☐	☐	☐
25	Helpless	☐	☐	☐	☐	☐
26	Weary	☐	☐	☐	☐	☐
27	Bewildered	☐	☐	☐	☐	☐
28	Furious	☐	☐	☐	☐	☐
29	Trusting	☐	☐	☐	☐	☐
30	Bad-Tempered	☐	☐	☐	☐	☐
31	Worthless	☐	☐	☐	☐	☐
32	Vigorous	☐	☐	☐	☐	☐
33	Uncertain about things	☐	☐	☐	☐	☐
34	Drained	☐	☐	☐	☐	☐
35	Enthusiastic	☐	☐	☐	☐	☐
		Not at all	A little	Moderately	Quite a bit	Extremely

Appendix 4: Eating behaviour screening questionnaires

Dutch Eating Behaviour Questionnaire (DEBQ)

1. If you have put on weight, do you eat less than you usually do?
Never ☐ Seldom ☐ Sometimes ☐ Often ☐ Very Often ☐
2. Do you try to eat less at mealtimes than you would like to eat?
Never ☐ Seldom ☐ Sometimes ☐ Often ☐ Very Often ☐
3. How often do you refuse food or drink offered because you are concerned about your weight?
Never ☐ Seldom ☐ Sometimes ☐ Often ☐ Very Often ☐
4. Do you watch exactly what you eat?
Never ☐ Seldom ☐ Sometimes ☐ Often ☐ Very Often ☐
5. Do you deliberately eat foods that are slimming?
Never ☐ Seldom ☐ Sometimes ☐ Often ☐ Very Often ☐
6. When you have eaten too much, do you eat less than usual the following days?
Never ☐ Seldom ☐ Sometimes ☐ Often ☐ Very Often ☐
7. Do you deliberately eat less in order not to become heavier?
Never ☐ Seldom ☐ Sometimes ☐ Often ☐ Very Often ☐
8. How often do you try not to eat between meals because you are watching your weight?
Never ☐ Seldom ☐ Sometimes ☐ Often ☐ Very Often ☐
9. How often in the evening do you try not to eat because you are watching your weight?
Never ☐ Seldom ☐ Sometimes ☐ Often ☐ Very Often ☐
10. Do you take into account your weight with what you eat?
Never ☐ Seldom ☐ Sometimes ☐ Often ☐ Very Often ☐

SCOFF Questionnaire

Please circle the response that applies:

Do you make yourself sick because you feel uncomfortably full?	Yes	No
Do you worry you have lost control over how much you eat?	Yes	No
Have you recently lost more than one stone in a 3 month period?	Yes	No
Do you believe yourself to be fat when others say you are too thin?	Yes	No
Would you say that food dominates your life?	Yes	No

Appendix 5: Taste test sheet

Food tasted

Spaghetti Bolognese	Mushroom Risotto
---------------------	------------------

Like
 Extremely
 Like very much
 Like moderately
 Like slightly
 Neither like nor dislike
 Dislike slightly
 Dislike moderately
 Dislike very much
 Dislike Extremely

Like
 Extremely
 Like very much
 Like moderately
 Like slightly
 Neither like nor dislike
 Dislike slightly
 Dislike moderately
 Dislike very much
 Dislike Extremely

Appendix 6: Visual analog scales used in Chapter 3

Please place a vertical mark along the line according to how you are feeling

Example: 

How hungry do you feel right now?

NOT AT ALL  EXTREMELY

How sick (or nauseous) do you feel right now?

NOT AT ALL  EXTREMELY

How pleasant would it be to eat right now?

NOT AT ALL  EXTREMELY

How much do you think you could eat right now?

NOTHING  A LARGE AMOUNT

How full do you feel right now?

NOT AT ALL  EXTREMELY

How sleepy do you feel right now?

NOT AT ALL  EXTREMELY

AFTER MEAL ONLY: How tasty was the meal?

NOT AT ALL  EXTREMELY

Appendix 7: Trait questionnaires used in Chapter 4

BIS/BAS Questionnaire – Page 1 /2

For each item, indicate how much you agree or disagree with what the item says. Please respond to all the items. Choose only one response to each statement. Please be as accurate and honest as you can be. Respond to each item as if it were the only item. That is, don't worry about being "consistent" in your responses. Choose from the following four response options:

1 = very true for me

2 = somewhat true for me

3 = somewhat false for me

4 = very false for me

1. A person's family is the most important thing in life. _____
2. Even if something bad is about to happen to me, I rarely experience fear or nervousness. _____
3. I go out of my way to get things I want. _____
4. When I'm doing well at something I love to keep at it. _____
5. I'm always willing to try something new if I think it will be fun. _____
6. How I dress is important to me. _____
7. When I get something I want, I feel excited and energized. _____
8. Criticism or scolding hurts me quite a bit. _____
9. When I want something I usually go all-out to get it. _____
10. I will often do things for no other reason than that they might be fun. _____
11. It's hard for me to find the time to do things such as get a haircut. _____
12. If I see a chance to get something I want I move on it right away. _____
13. I feel pretty worried or upset when I think or know somebody is angry at me. _____

BIS/BAS Questionnaire – Page 2 / 2

For each item, indicate how much you agree or disagree with what the item says. Please respond to all the items. Choose only one response to each statement. Please be as accurate and honest as you can be. Respond to each item as if it were the only item. That is, don't worry about being "consistent" in your responses. Choose from the following four response options:

- 1 = very true for me**
- 2 = somewhat true for me**
- 3 = somewhat false for me**
- 4 = very false for me**

- 14. When I see an opportunity for something I like I get excited right away. _____
- 15. I often act on the spur of the moment. _____
- 16. If I think something unpleasant is going to happen I usually get pretty "worked up." _____
- 17. I often wonder why people act the way they do. _____
- 18. When good things happen to me, it affects me strongly. _____
- 19. I feel worried when I think I have done poorly at something important. _____
- 20. I crave excitement and new sensations. _____
- 21. When I go after something I use a "no holds barred" approach. _____
- 22. I have very few fears compared to my friends. _____
- 23. It would excite me to win a contest. _____
- 24. I worry about making mistakes. _____

SELF-EVALUATION QUESTIONNAIRE

STAI Form Y-2

DIRECTIONS

A number of statements which people have used to describe themselves are given below. Read each statement and then circle the appropriate number to the right of the statement to indicate how you *generally* feel. There are no right or wrong answers. Do not spend too much time on any one statement but give the answer which seems to describe how you generally feel.

ALMOST NEVER
SOMETIMES
OFTEN
ALMOST ALWAYS

- | | | | | |
|--|---|---|---|---|
| 21. I feel pleasant..... | 1 | 2 | 3 | 4 |
| 22. I feel nervous and restless | 1 | 2 | 3 | 4 |
| 23. I feel satisfied with myself..... | 1 | 2 | 3 | 4 |
| 24. I wish I could be as happy as others seem to be | 1 | 2 | 3 | 4 |
| 25. I feel like a failure | 1 | 2 | 3 | 4 |
| 26. I feel rested | 1 | 2 | 3 | 4 |
| 27. I am "calm, cool, and collected" | 1 | 2 | 3 | 4 |
| 28. I feel that difficulties are piling up so that I cannot overcome them..... | 1 | 2 | 3 | 4 |
| 29. I worry too much over something that really doesn't matter..... | 1 | 2 | 3 | 4 |
| 30. I am happy | 1 | 2 | 3 | 4 |
| 31. I have disturbing thoughts | 1 | 2 | 3 | 4 |
| 32. I lack self-confidence..... | 1 | 2 | 3 | 4 |
| 33. I feel secure | 1 | 2 | 3 | 4 |
| 34. I make decisions easily | 1 | 2 | 3 | 4 |
| 35. I feel inadequate..... | 1 | 2 | 3 | 4 |
| 36. I am content | 1 | 2 | 3 | 4 |
| 37. Some unimportant thought runs through my mind and bothers me | 1 | 2 | 3 | 4 |
| 38. I take disappointments so keenly that I can't put them out of my mind | 1 | 2 | 3 | 4 |
| 39. I am a steady person..... | 1 | 2 | 3 | 4 |
| 40. I get in a state of tension or turmoil as I think over my recent concerns
and interests | 1 | 2 | 3 | 4 |

Subjective Happiness Scale

Instructions: For each of the following statements and/or questions, please circle the point on the scale that you feel is most appropriate in describing you.

1. In general, I consider myself:

not a very happy person 1 2 3 4 5 6 7 a very happy person

2. Compared to most of my peers, I consider myself:

less happy 1 2 3 4 5 6 7 more happy

3. Some people are generally very happy. They enjoy life regardless of what is going on, getting the most out of everything. To what extent does this characterization describe you?

not at all 1 2 3 4 5 6 7 a great deal

4. Some people are generally not very happy. Although they are not depressed, they never seem as happy as they might be. To what extent does this characterization describe you?

not at all 1 2 3 4 5 6 7 a great deal

Satisfaction with Life Scale

Instructions: Below are five statements that you may agree or disagree with. Using the 1 - 7 scale below, indicate your agreement with each item by placing the appropriate number on the line preceding that item. Please be open and honest in your responding.

- 7 - Strongly agree
- 6 - Agree
- 5 - Slightly agree
- 4 - Neither agree nor disagree
- 3 - Slightly disagree
- 2 - Disagree
- 1 - Strongly disagree

_____ In most ways my life is close to my ideal.

_____ The conditions of my life are excellent.

_____ I am satisfied with my life.

_____ So far I have gotten the important things I want in life.

_____ If I could live my life over, I would change almost nothing.

The Three-Factor Eating Questionnaire

Please read each statement and select from the answer that indicates the frequency with which you find yourself feeling or experiencing what is being described in the statements below.

1. When I smell a delicious food, I find it very difficult to keep from eating, even if I have just finished a meal.	Definitely True	Mostly True	Mostly False	Definitely False
2. I deliberately take small helpings as a means of controlling my weight.	Definitely True	Mostly True	Mostly False	Definitely False
3. When I feel anxious, I find myself eating.	Definitely True	Mostly True	Mostly False	Definitely False
4. Sometimes when I start eating, I just can't seem to stop.	Definitely True	Mostly True	Mostly False	Definitely False
5. Being with someone who is eating often makes me hungry enough to eat also.	Definitely True	Mostly True	Mostly False	Definitely False
6. When I feel blue, I often overeat.	Definitely True	Mostly True	Mostly False	Definitely False
7. When I see a real delicacy, I often get so hungry that I have to eat right away.	Definitely True	Mostly True	Mostly False	Definitely False
8. I get so hungry that my stomach often seems like a bottomless pit.	Definitely True	Mostly True	Mostly False	Definitely False
9. I am always hungry so it is hard for me to stop eating before I finish the food on my plate.	Definitely True	Mostly True	Mostly False	Definitely False
10. When I feel lonely, I console myself by eating.	Definitely True	Mostly True	Mostly False	Definitely False
11. I consciously hold back at meals in order not to gain weight.	Definitely True	Mostly True	Mostly False	Definitely False
12. I do not eat some foods because they make me fat.	Definitely True	Mostly True	Mostly False	Definitely False
13. I am always hungry enough to eat at any time.	Definitely true	Mostly true	Mostly False	Definitely False
14. How often do you feel hungry?	Almost Always	Often between meals	Sometimes between meals	Only at mealtimes
15. How frequently do you avoid "stocking up" on tempting foods?	Almost Always	Usually	Seldom	Almost never
16. How likely are you to consciously eat less than you want?	Very Likely	Moderately Likely	Slightly Likely	Unlikely
17. Do you go on eating binges even though you are not hungry?	At least once a week	Sometimes	Rarely	Never
18. On a scale of 1 to 8, where 1 means no restraint in eating (eating whatever you want, whenever you want it) and 8 means total restraint (constantly limiting food intake and never "giving in"), what number would you give yourself?				
/8				

Appendix 8: State questionnaires used in Chapter 4

Food Cravings Questionnaire – State (FCQ-S15)

Please read each of the following statements carefully and select the choice that best describes how you feel **RIGHT NOW**.

		Strongly Disagree	Disagree	Neither Agree nor Disagree	Agree	Strongly Agree
1.	I'm craving food					
2.	I have an urge for food					
3.	I have an intense desire to eat something					
4.	If I ate something, I wouldn't feel so sluggish and lethargic					
5.	Satisfying my appetite would make me feel less grouchy and irritable					
6.	I would feel more alert if I could satisfy my appetite					
7.	If I ate right now, my stomach wouldn't feel as empty					
8.	I am hungry					
9.	I feel weak because of not eating					
10.	My desire to eat something seems overpowering					
11.	I know I'm going to keep on thinking about food until I actually have it					
12.	If I had something to eat, I would not be able to stop eating it					
13.	If I were to eat something, I am sure my mood would improve					
14.	Eating something would feel wonderful					
15.	Eating something would make things just perfect					

Visual Analog Scale

Please place a vertical mark along the line according to how you are feeling

Example: 

How hungry do you feel right now?

NOT AT ALL  EXTREMELY

How sick (or nauseous) do you feel right now?

NOT AT ALL  EXTREMELY

How pleasant would it be to eat right now?

NOT AT ALL  EXTREMELY

How much do you think you could eat right now?

NOTHING  A LARGE AMOUNT

How full do you feel right now?

NOT AT ALL  EXTREMELY

How sleepy do you feel right now?

NOT AT ALL  EXTREMELY

Publications & Communications

Directly arising from this thesis:

Publications (please see attached at back of thesis)

Kisspeptin enhances brain responses to olfactory and visual cues of attraction in men.

Yang, L.*, Demetriou, L.*, Wall, M.B., Mills, E.G., Zargaran, D., Sykes, M., Prague, J.K., Abbara, A., Owen, B.M., Bassett, P.A., Rabiner, E.A., Comninou, A.N., Dhillon, W.S.

JCI Insight. 2020; 5 (3). * joint first authors

The effects of kisspeptin on β -cell function, serum metabolites and appetite in humans.

Izzi-Engbeaya, C., Comninou, A.N., Clarke, S.A., Jomard, A., **Yang, L.**, Jones, S., Abbara, A., Narayanaswamy, S., Eng, P.C., Papadopoulou, D., Prague, J.K., Bech, P., Godsland, I.F., Bassett, P., Sands, C., Camuzeaux, S., Gomez-Romero, M., Pearce, J.T.M., Lewis, M.R., Holmes, E., Nicholson, J.K., Tan, T., Ratnasabapathy, R., Hu, M., Carrat, G., Piemonti, L., Bugliani, M., Marchetti, P., Johnson, P.R., Hughes, S.J., James Shapiro, A.M., Rutter, G.A., Dhillon, W.S. Diabetes, Obes. Metab. 2018; 20 (12), 2800–2810.

Oral Communications

Kisspeptin enhances brain processing of olfactory and visual cues of attraction in men.

Yang, L., Demetriou, L., Wall, M.B., Mills, E.G., Zargaran, D., Sykes, M., Prague, J.K., Abbara, A., Owen, B.M., Bassett, P.A., Rabiner, E.A., Comninou, A.N., Dhillon, W.S.

American Endocrine Society Annual Conference, March 2019, New Orleans

Outstanding Abstract Award

Kisspeptin enhances the brain processing of attraction in men.

Yang, L., Demetriou, L., Wall, M.B., Mills, E.G., Zargaran, D., Sykes, M., Prague, J.K., Abbara, A., Owen, B.M., Bassett, P.A., Rabiner, E.A., Comninou, A.N., Dhillon, W.S.

Society for Endocrinology Annual Conference, November 2019, Brighton

Awarded Best Reproductive Endocrinology and Biology Oral Communication Prize

Other related publications during my PhD studies:

Original Papers

Effects of Glucagon-like Peptide-1 on the Reproductive Axis in Healthy Men.

Izzi-Engbeaya, C., Jones, S., Crustna, Y., Machenahalli, P.C., Papadopoulou, D., Modi, M., Panayi, C., Starikova, J., Eng, P.C., Phylactou, M., Mills, E., **Yang, L.**, Ratnasabapathy, R., Sykes, M., Plumptre, I., Coumbe, B., Wing, V.C., Pacuszka, E., Bech, P., Minnion, J., Tharakan, G., Tan, T., Veldhuis, J., Abbara, A., Comninou, A.N., Dhillon, W.S.

J. Clin. Endocrinol. Metab. 2020; (105) 4.

Effects of Peptide YY on the Hypothalamic-Pituitary-Gonadal Axis in Healthy Men.

Izzi-Engbeaya, C., Jones, S., Crustna, Y., Machenahalli, P.C., Papadopoulou, D., Modi, M., Panayi, C., Starikova, J., Eng, P.C., Phylactou, M., Mills, E., **Yang, L.**, Ratnasabapathy, R., Sykes, M., Plumptre, I., Coumbe, B., Wing, V., Pacuszka, E., Bech, P., Minnion, J., Tharakan, G., Tan, T., Veldhuis, J., Abbara, A., Comninou, A.N., Dhillon, W.S.

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In: Model Animals in Neuroendocrinology 2018. John Wiley & Sons Ltd, UK, pp. 383–405.

Kisspeptin enhances brain responses to olfactory and visual cues of attraction in men

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Successful reproduction is a fundamental physiological process that relies on the integration of sensory cues of attraction with appropriate emotions and behaviors and the reproductive axis. However, the factors responsible for this integration remain largely unexplored. Using functional neuroimaging, hormonal, and psychometric analyses, we demonstrate that the reproductive hormone kisspeptin enhances brain activity in response to olfactory and visual cues of attraction in men. Furthermore, the brain regions enhanced by kisspeptin correspond to areas within the olfactory and limbic systems that govern sexual behavior and perception of beauty as well as overlap with its endogenous expression pattern. Of key functional and behavioral significance, we observed that kisspeptin was most effective in men with lower sexual quality-of-life scores. As such, our results reveal a previously undescribed attraction pathway in humans activated by kisspeptin and identify kisspeptin signaling as a new therapeutic target for related reproductive and psychosexual disorders.

Introduction

Attraction to another individual is the fundamental initiating step for sexual behavior (1). This innate process relies on the integration of multimodal sensory cues with appropriate emotional and behavioral outputs. However, the intrinsic factors that mediate human attraction remain incompletely understood. Unraveling these integral processes is of major scientific and clinical importance, as related psychosexual disorders affect up to 1 in 3 people worldwide (2, 3), with significant detrimental effects on quality of life, interpersonal relationships, and fertility (4). Furthermore, despite the high clinical burden, our limited understanding of the brain processes governing human psychosexual function has restricted the development of effective treatments (5).

Olfactory and visual signals provide key sensory inputs for sexual attraction. Olfaction is central to many important sexual behaviors, and its role in mate attraction has been extensively studied in nonhuman mammals (6). Olfactory function is also significantly correlated with sexual function in humans (7, 8), and the primary olfactory network has numerous projections to key limbic areas involved in sexual and emotional processing (9). Furthermore, functional neuroimaging has demonstrated that when exposed to a feminine scent, heterosexual men exhibit increased brain activity in limbic regions associated with sexual desire and arousal (10). The visual appreciation of beauty is another fundamental aspect of human attraction, with evidence that prefrontal areas, in particular the medial prefrontal cortex (mPFC), are heavily involved in the human perception of beauty (11–13). Facial beauty provides a symbol of sexual potential, with evidence that men show consistent cross-cultural preferences for certain female facial characteristics (14). Thus, studying factors that may modulate these olfactory and visual cues of attraction can provide important insights into human psychosexual function.

Authorship note: LY and LD are co-first authors. ANC and WSD are co-senior authors.

Conflict of interest: The authors have declared that no conflict of interest exists.

Submitted: September 18, 2019

Accepted: December 18, 2019

Published: February 13, 2020.

Reference information: *JCI Insight*. 2020;5(3):e133633. <https://doi.org/10.1172/jci.insight.133633>.

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The intrinsic factors that integrate and coordinate these fundamental aspects of human attraction with limbic and reproductive pathways remain as yet unknown. The reproductive hormone kisspeptin is a crucial endogenous activator of the reproductive axis (15–20) and is widely expressed in limbic brain regions along with its cognate receptor (21–24). Furthermore, kisspeptin neurons interconnect olfactory, limbic, and hypothalamic reproductive centers in rodents (25). Studies in animals suggest that kisspeptin influences brain pathways controlling reproductive behaviors (26–28) and is requisite for olfactory system-mediated partner preference (28, 29). Combined, these data led us to postulate that kisspeptin may be an elusive factor in human attraction, which has not been investigated until now to our knowledge. We therefore hypothesized that kisspeptin enhances the brain processing of attraction in humans.

To test our hypothesis, we performed a randomized, double-blind, 2-way crossover, placebo-controlled study in 33 healthy, heterosexual men (mean age 24.5 ± 0.7 years, mean BMI 22.9 ± 0.8 kg/m²) using functional neuroimaging, hormonal, and psychometric assessments to examine the effects of peripheral kisspeptin (via intravenous infusion) on brain processing during olfactory and facial attractiveness tasks (Figure 1A).

Results

Kisspeptin administration increased circulating kisspeptin but not testosterone or cortisol levels. At baseline (pre-infusion), kisspeptin, gonadotropin, and testosterone levels were equivalent between study visits (Supplemental Table 2; supplemental material available online with this article; <https://doi.org/10.1172/jci.insight.133633DS1>). Subsequently, intravenous kisspeptin infusion (1 nmol/kg/h) significantly increased circulating kisspeptin levels, reaching steady state for the duration of fMRI scanning and psychometric questionnaires (Figure 1B). As expected, kisspeptin administration led to raised luteinizing hormone (LH) levels (Supplemental Figure 1), which indicated that the dose of kisspeptin used was biologically active. We also took precautions to avoid other hormonal confounders. Indeed, kisspeptin administration had no significant effects on testosterone levels during the 75-minute study period because each study was completed before any downstream increases in testosterone (Supplemental Figure 1), which have previously been shown to occur following longer periods of kisspeptin exposure in humans (30). Baseline cortisol levels were also equivalent between study visits, and kisspeptin administration had no significant effects on cortisol levels (Supplemental Figure 1 and Supplemental Table 2).

Kisspeptin enhanced brain activity in olfactory and limbic circuits governing human sexual behavior, on exposure to a pleasant feminine scent. During the olfactory task, a feminine scent was delivered nasally, alternating with odorless air as baseline. Chanel No5 was selected as the olfactory stimulus because it has previously been shown to activate limbic regions associated with sexual arousal in a validated fMRI protocol (10). Consistent with this, participants in the current study also validated it as a pleasant and feminine scent (Supplemental Table 1). We observed that kisspeptin significantly enhanced brain activity compared with placebo in key limbic areas related to olfaction and sexual processing (including the amygdala and thalamus), on whole-brain voxel-wise analysis, in response to this pleasant feminine scent (Figure 2A and Supplemental Table 4).

To explore this further, we performed a regions of interest (ROI) analysis (Figure 2B) with a priori-defined regions involved in olfactory and sexual processing and brain regions known to express kisspeptin receptors in humans (9, 21, 31). Kisspeptin significantly increases brain activity in the amygdala, a central component of the primary olfactory cortex (9), as well as the hippocampus, insula, and orbitofrontal cortex (OFC), which form key limbic projections from the primary olfactory cortex and are common substrates for olfactory and emotional processing (9, 31). Furthermore, kisspeptin significantly enhanced activity in the globus pallidus and putamen, which constitute part of the olfactory hedonic processing network, along with the amygdala, hippocampus, and OFC (32). Interestingly, regions associated with reward, motivation, and “romantic love” were also enhanced by kisspeptin, including the thalamus, posterior cingulate cortex (PCC), and caudate (33, 34). Moreover, kisspeptin’s enhancement of thalamus and insula activity corresponded to these established areas of activation during physiological sexual arousal (35). Collectively, these data indicate that kisspeptin augments olfactory as well as sexual and emotional processing in response to pleasurable olfactory stimuli in men.

Next, we used a systems-based approach to investigate the overall effects of kisspeptin on brain systems controlling olfaction and sexual arousal. Using brain masks derived from meta-analytic data (36), we performed a secondary ROI analysis of overall kisspeptin effect compared with placebo on the olfactory system and sexual arousal system, with the motor system as a control. Here, kisspeptin significantly enhanced

brain activity in both the olfactory and sexual arousal systems but not in the control motor system, which highlights the specificity of kisspeptin's effects (Figure 2C).

Kisspeptin enhanced brain activity in areas governing the evaluation of beauty, on viewing female faces. To investigate the effects of kisspeptin on the perception of facial beauty, participants were presented with 60 female faces in random order, selected from a validated database, comprising 3 groups of 20 faces rated in accordance with attractiveness (high, medium, low) by 1087 independent raters (37). Participants viewing faces rated to have high and medium attractiveness exhibited enhanced activity in the mPFC and the superior frontal gyrus (SFG) during kisspeptin administration compared with placebo on whole-brain voxel-wise analyses (Figure 3, B and C, and Supplemental Table 4). Consistent with our data, the mPFC is a well-established area involved in the appreciation of facial beauty (11, 12). In addition, functional neuroimaging has also demonstrated SFG enhancement in response to attractive faces (38), and both the mPFC and SFG express kisspeptin receptors in humans (21, 22). We therefore undertook ROI analyses of both regions to explore this further. In keeping with the literature, graded responses in overall mPFC and SFG activity were observed with increasing facial attractiveness in the placebo groups (Figure 3, D and E) (12, 38). Remarkably, kisspeptin significantly enhanced both mPFC and SFG activity compared with placebo across all 3 categories of attractiveness (Figure 3, D and E). Together, these findings demonstrate that kisspeptin augments the processing of facial beauty across a spectrum of facial attractiveness, therefore serving as an amplifier within the human neural aesthetic circuitry involved in the assessment of facial beauty.

We also explored areas known to express kisspeptin receptors and limbic regions involved in sexual arousal by performing an ROI analysis based on a priori-defined brain regions (accumbens, amygdala, anterior cingulate cortex, caudate, globus pallidus, hippocampus, PCC, putamen, and thalamus) (21, 39). Our results revealed that kisspeptin's enhancement of brain activity on viewing a female face was specific to the mPFC and SFG (established aesthetic regions) because kisspeptin did not significantly modulate brain activity in these other regions (Supplemental Figure 2).

The effects of kisspeptin on brain activity were more pronounced in men with lower baseline reward and sexual quality of life. To assess behavioral and functional relevance for our brain activity data, participants also completed standardized, validated psychometric questionnaires (Supplemental Tables 1 and 2), including questionnaires designed to assess reward and sexual quality of life (40–43). Correlation analyses between these behavioral parameters and neuroimaging data demonstrated that participants with lower baseline behavioral activation system (BAS) reward scores (40) showed greater kisspeptin-enhanced brain activity in the PCC, on viewing faces rated high ($r = -0.487$, and $P = 0.004$, Figure 3G) and medium attractiveness ($r = -0.463$, and $P = 0.007$, Figure 3F). Similarly, participants who reported a lower baseline sexual quality of life score (43) showed much greater kisspeptin-enhanced activity in the anterior cingulate cortex ($r = -0.414$, and $P = 0.01$) and insula ($r = -0.441$, and $P = 0.01$) on viewing faces with low attractiveness (Figure 3, H and I), key regions involved in reward and incentive motivation (44, 45). These behavioral findings therefore provide crucial relevance for our brain activity data and lay the foundation for future clinical applications of kisspeptin.

Discussion

In this study, we demonstrate for the first time to our knowledge that the reproductive hormone kisspeptin enhances brain activity specifically in response to olfactory and visual cues of attraction in healthy men. During the olfactory task, areas significantly enhanced by kisspeptin included key limbic regions known to be involved in human olfactory processing and sexual arousal, but crucially, kisspeptin did not affect the motor system, which was used as a control. Similarly, during the facial attractiveness task, kisspeptin selectively amplified the mPFC and SFG (established aesthetic regions) (Figure 3, A–C). In contrast, other limbic regions were unaffected by kisspeptin during this facial attractiveness task (Supplemental Figure 2), unlike in the olfactory task (Figure 2B). These data highlight our findings of targeted region-specific effects of kisspeptin, dependent on the nature of the attraction cue (olfactory or visual).

Studies in different animal species have shown varying anatomical expression patterns for kisspeptin and its receptor, which may be due to species and methodological differences (23, 24). Therefore, to select brain regions of interest for our fMRI analyses, we used the available data describing distribution of human kisspeptin receptor mRNA (21, 22). Our results show that peripheral kisspeptin administration enhanced brain regions matching areas where human kisspeptin receptor is expressed, suggesting a direct receptor-mediated action of kisspeptin in these brain regions. Given these findings, it is important to consider how peripherally administered kisspeptin can reach brain regions of interest. Gonadotropin-releasing hormone (GnRH) neurons

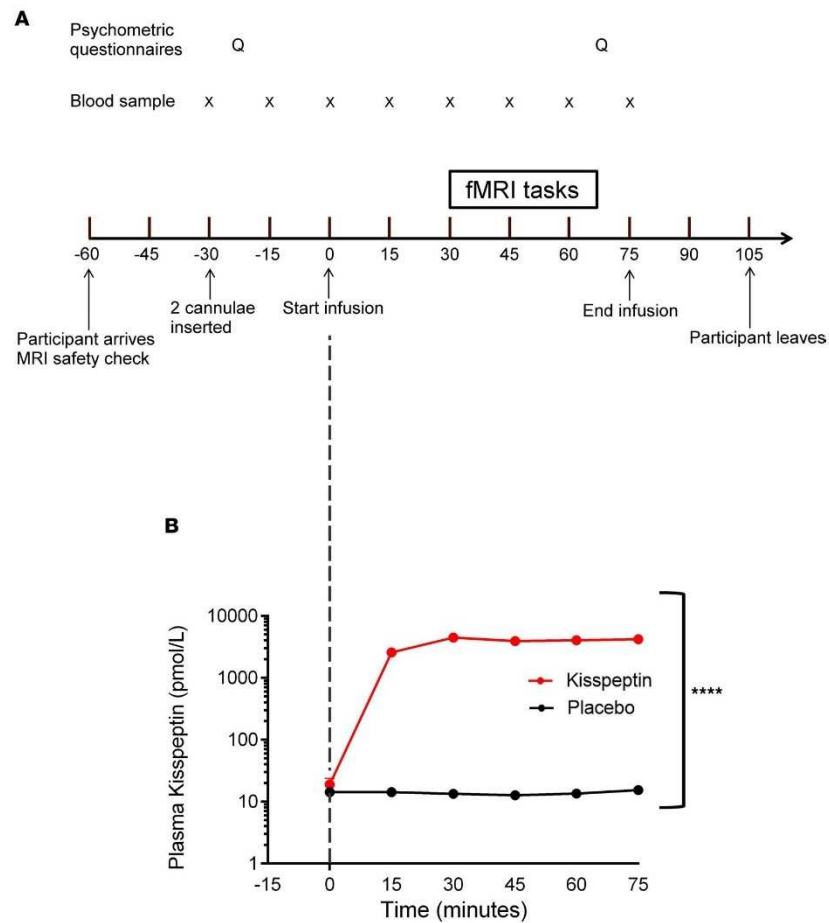
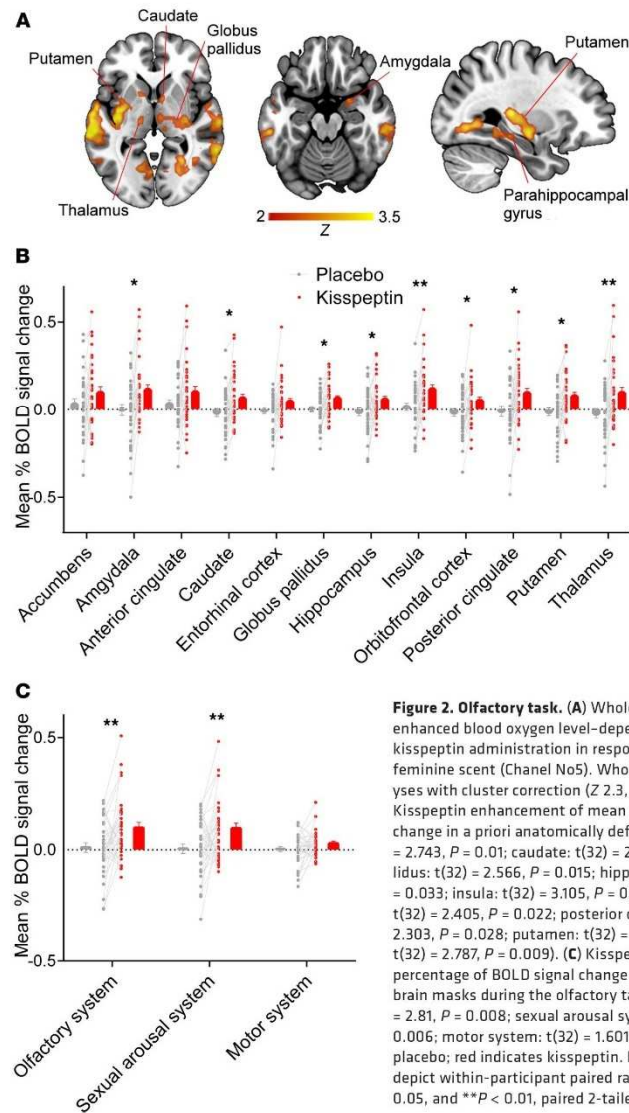


Figure 1. Experimental protocol and effects of kisspeptin administration on circulating kisspeptin levels. (A) Thirty-three healthy young men participated in a randomized, double-blind, 2-way crossover, placebo-controlled study. They attended 2 study visits: 1 for intravenous administration of kisspeptin (1 nmol/kg/h) and 1 for intravenous administration of an equivalent volume of placebo (vehicle) for 75 minutes. Blood samples were taken every 15 minutes (X). Participants completed baseline and intra-infusion psychometric questionnaires (Q) and underwent functional MRI (fMRI) scanning while performing olfactory and facial attractiveness tasks. **(B)** Kisspeptin infusion resulted in increased circulating kisspeptin levels (**** $P < 0.0001$, and $n = 33$), reaching a plateau at 30 minutes after initiation, with stable circulating kisspeptin levels during the fMRI and intra-infusion psychometric questionnaires.

extend dendritic terminals beyond the blood-brain barrier; thus, peripheral kisspeptin is capable of stimulating GnRH, and in turn, LH, without needing to cross the blood-brain barrier (19). However, different isoforms of kisspeptin have been shown to have varying degrees of blood-brain barrier penetrance. Peripherally administered kisspeptin-54, as used in this study, is capable of reaching both GnRH cell bodies beyond the blood-brain barrier (46) as well as numerous limbic brain structures known to express kisspeptin and its receptor (39).

It is also important to consider additional pathways that may be involved in exerting the observed downstream effects of kisspeptin on the brain. Kisspeptin activates GnRH neurons, and these have been identified in several areas of the brain in humans, including the cerebellum, thalamus, anterior olfactory areas, amygdala, striatum, ventral pallidum, and putamen (47–49). Interestingly, we identified enhanced brain activity



due to kisspeptin administration in several areas that are not known to contain GnRH neurons or GnRH receptors in humans (including the caudate, globus pallidus, insula, and PCC) (47–50), which suggests there are GnRH-independent actions of kisspeptin in these regions. Furthermore, there is recent functional evidence that certain kisspeptin-induced sexual behaviors can occur independent of GnRH. These include kisspeptin-induced lumbar lordosis in mice (28) and kisspeptin-stimulated erections in rats (26). In addition, data from animal studies demonstrate that kisspeptin also interacts with numerous other neuropeptide systems, including serotonin (51, 52), dopamine (25), vasopressin (25), GABA (53), glutamate (54), and nitric oxide (16, 28, 55). Thus, the fMRI changes that we have shown may be the product of direct kisspeptin effects on its receptor as well as interactions between kisspeptin and these other downstream neural systems.

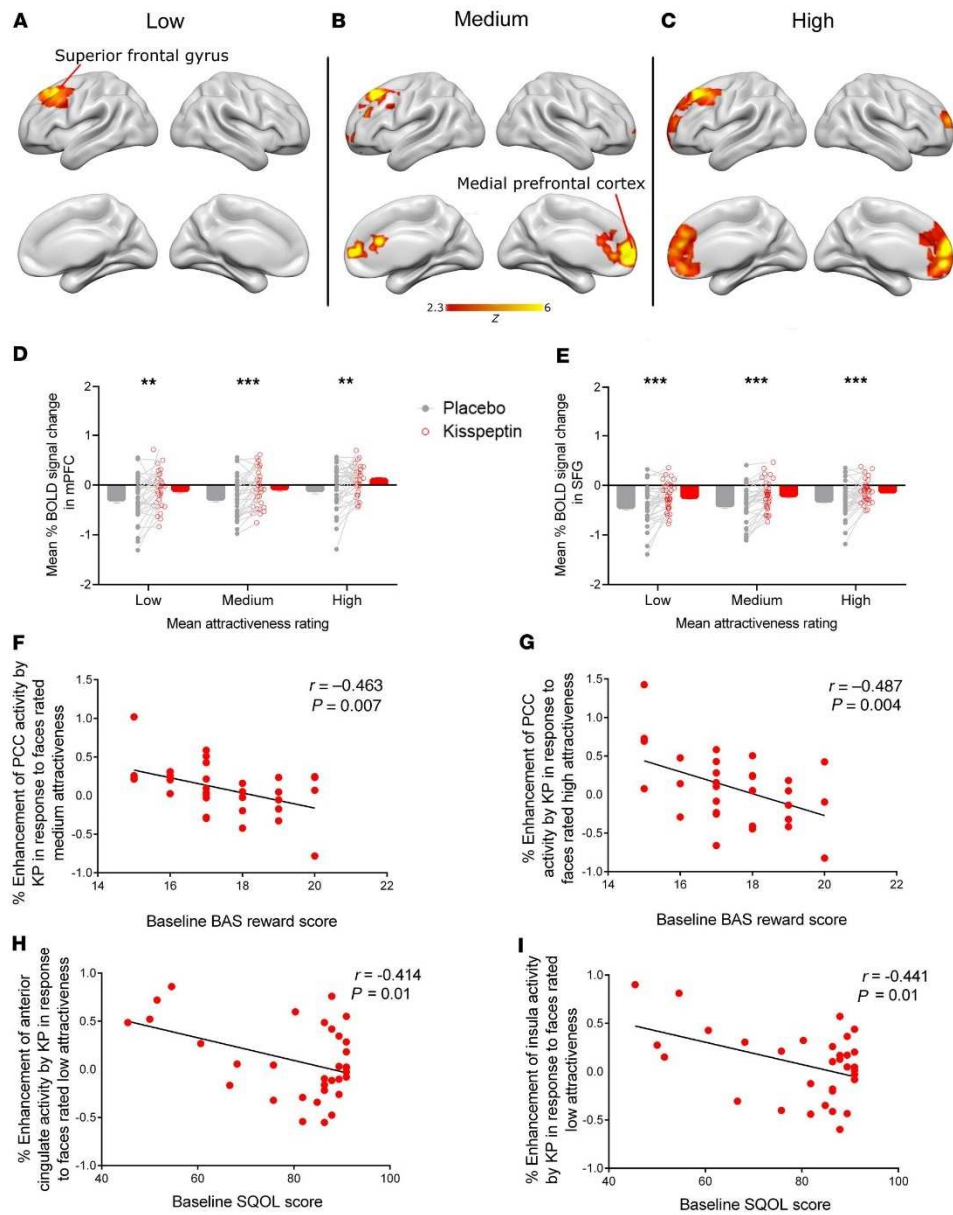


Figure 3. Facial attractiveness task. (A–C) Whole-brain analysis of kisspeptin-enhanced BOLD activity in response to faces rated (A) low, (B) medium, and (C) high attractiveness ($n = 33$). (D) Mean percentage of BOLD signal change in the functionally defined ROI, mPFC, low: $t(32) = 2.804$, $P = 0.009$; medium: $t(32) = 4.966$, $P < 0.001$; high: $t(32) = 3.066$, $P = 0.004$. (E) Mean percentage of BOLD signal change in the functionally defined ROI, SFG, low: $t(32) = 3.966$, $P < 0.001$; medium: $t(32) = 4.567$, $P < 0.001$; high: $t(32) = 3.668$, $P < 0.001$. (** $P < 0.01$, and *** $P < 0.001$, paired 2-tailed t test.) (F and G) Pearson's correlation between BAS reward score and PCC enhancement by kisspeptin in response to faces rated (F) medium ($r = -0.463$, and $P = 0.007$) and (G) high ($r = -0.487$, and $P = 0.004$) attractiveness. (H and I) Pearson's correlation between sexual quality-of-life (SQOL) score and enhancement of (H) ACC ($r = -0.414$, and $P = 0.01$) and (I) insula ($r = -0.441$, and $P = 0.01$) activity by kisspeptin in response to faces of low attractiveness ($n = 33$).

In this study, we have shown that kisspeptin administration can enhance brain processing in olfactory and sexual arousal systems of healthy men in response to a validated olfactory cue. In animal studies, opposite-sex odors or pheromones have been used to elicit olfactory system-driven behavioral and endocrine responses (28, 56). In humans, the evidence for behavioral and physiological responses to opposite-sex odors is conflicting (57); therefore, we selected Chanel No5 as a widely recognized and validated feminine scent (10) to activate brain areas responding to a consciously perceived female olfactory cue so that kisspeptin's effects on these regions could be robustly assessed. Our results show that kisspeptin administration enhances brain responses to a feminine scent in several key components of the limbic system that are involved in olfactory processing, hedonic valuation of olfactory stimuli, and sexual arousal networks (9, 31). Intriguingly, the same enhancement in limbic activity is not seen during the facial attractiveness task. Instead, kisspeptin significantly enhanced activity in the mPFC and SFG, 2 prefrontal regions that are known to govern the perception of facial beauty (11, 12, 38). This is in keeping with previous data indicating that limbic reward pathways are not necessarily involved in facial aesthetic assessment (11). Thus, our data demonstrate that kisspeptin's effects on brain activity are specific to the relevant regions involved in different sensory modalities of attraction (olfactory or visual).

Furthermore, we observed significant correlations between kisspeptin-enhanced brain activity and important psychometric parameters, thereby providing key behavioral and functional relevance for the observed brain changes. Indeed, greater kisspeptin enhancement was observed in the PCC on viewing attractive faces in men with lower baseline reward scores. The PCC is implicated in romantic love (34), and its activity is known to vary with emotional memory and reward (58). Thus, kisspeptin may act to enhance emotional salience and reward processing in the PCC on viewing attractive faces in order to rebalance a lower reward drive in these individuals, in favor of promoting sexual attraction. A similar and perhaps even more intriguing relationship was demonstrated between kisspeptin's enhancement of ACC and insula activity in response to female faces and low sexual quality of life. The putamen is a dopamine-rich area that responds to visual sexual stimuli (59), and both the ACC and insula are areas implicated in sexual arousal (60), facial attraction (12, 38), and motivation toward reward (44, 45). Thus kisspeptin's enhancement of these brain regions on viewing attractive faces may serve to strengthen feelings of reward, attraction, and incentive motivation in individuals experiencing lower sexual quality of life, ultimately to encourage reproduction at a population level. Collectively, these findings provide key behavioral and functional relevance for kisspeptin's enhancement of brain activity on viewing attractive faces and lay the foundation for potential clinical applications of our data for patients with common reproductive and psychosexual disorders.

We demonstrate these effects on human brain processing through the administration of kisspeptin, achieving plasma kisspeptin levels similar to the levels required to restore physiological LH pulsatility in women with hypothalamic amenorrhoea (61). Equivalent plasma concentrations of kisspeptin are also observed physiologically in normal pregnancy (62). Although kisspeptin can modulate GnRH pulsatility in humans (63–65), current evidence suggests that its effects on behavior do not rely on changes in GnRH pulsatility because LH remained elevated and nonpulsatile in this as well as previous work showing kisspeptin-induced behavioral changes (39). In addition and as discussed earlier, kisspeptin exerts certain GnRH-independent behavioral effects, and we have previously seen a reduction in negative mood and sexual aversion without any observed modulations of LH pulsatility (39). Taken together, these data suggest that the behavioral effects of kisspeptin can be achieved without modulation of downstream GnRH pulsatility. As such, this introduces a new and exciting avenue for kisspeptin therapeutics in the management of psychosexual disorders in addition to common reproductive disorders, with further studies necessary to fully elucidate the role of endogenous kisspeptin activity.

When considering attraction, it is also important to bear in mind that although visual and olfactory inputs are fundamental cues of attraction in many species (1), human sexual responses are complex and influenced by multiple additional components, such as context, personality traits, and body language (66), which may affect individual perceptions of attractiveness. In our study, we controlled for some of these factors by omitting motion picture and audio stimuli as potential confounders in participant responses; however, it would be interesting to study these other components of attraction in the future. In addition, we ensured that only investigators of the same sex conducted physical interactions with participants during the study (e.g., administering psychometric questionnaires, cannulation, and blood sampling) to standardize any effects of experimenter sex (67). Importantly, we also took precautions to measure related

hormones that could confound our results. Indeed, we observed that kisspeptin had no effect on cortisol or testosterone levels during this time course (Supplemental Figure 1).

In summary, our findings reveal a previously undescribed kisspeptin-activated attraction pathway, uniting 2 major sensory inputs of human attraction with corresponding neural processing and reproductive hormonal control. Furthermore, kisspeptin's enhancement of key brain areas within these pathways is augmented in men with lower behavioral reward drive and sexual quality of life, providing crucial functional relevance to our imaging findings. Collectively, this has important implications for our understanding of human psychosexual function and its endocrine control. Crucially, our data also lay the foundation for manipulation of these newly identified kisspeptin-mediated pathways to deliver much-needed clinical strategies for individuals suffering from related common reproductive and psychosexual disorders.

Methods

Participants. Previous work demonstrates that kisspeptin enhances task-based brain activity, measured by percentage of BOLD signal change, in the amygdala by mean 0.74% and standard deviation 0.38% compared with placebo (mean 0.48%, standard deviation 0.51%) (39), and we expected a similar response in this study. Using these data, with α 0.05, with power 0.8, and assuming correlation between means of 0.40, a power calculation was performed, resulting in a sample size of 31. This is in line with previous fMRI studies and empirically derived estimates of optimal sample sizes in fMRI studies (39, 68, 69). To allow for natural variation in responses, dropouts, and exclusions, 36 participants were recruited via advertisements. One participant withdrew, 1 participant did not complete the fMRI tasks, and 1 participant was excluded due to a change in health status during the study, giving a final study group of 33 healthy young men. To confirm eligibility, participants attended a medical screening appointment. Participants were free of current and past physical or psychiatric illness and were naive to psychoactive substances, prescribed or illicit. Heterosexuality was determined by a Kinsey score of 0 (70), and normal olfactory function was assessed by the Brief Smell Identification Test (71). All participants had normal basal reproductive hormone levels (Supplemental Table 1). Specific screening was also undertaken to exclude participants with any history of sexual aggression/abuse/phobia or psychotherapy/counseling. In addition, tobacco smokers were excluded because smoking is associated with olfactory deficits that could interfere with the olfactory fMRI task (72). All participants were right-handed and had normal or corrected-to-normal vision.

Study design. The participants completed 2 MRI study visits each, as part of a randomized, double-blind, 2-way crossover, placebo-controlled protocol. Therefore, this was a within-participant design study, in which the participants acted as their own controls, thereby minimizing variability and enhancing power. All the studies commenced in the morning to control for time-dependent hormonal changes. Participants were asked to abstain from sexual activities from midnight before the study visit because prior sexual activity can affect testosterone levels (73, 74). In addition, participants were asked to abstain from alcohol and caffeine for the same period of time and to consume a normal breakfast on the morning of their study visit.

On arrival, participants were asked to change into loose hospital scrubs and relax in a supine position for 30 minutes. Intravenous cannulae were then inserted into each antecubital fossa for infusion of kisspeptin or placebo and blood collection (at time points -30, -15, 0, 15, 30, 45, 60, and 75 minutes) (Figure 1A). Participants also completed psychometric questionnaires as detailed below. At time point 0 an infusion of kisspeptin or placebo was commenced lasting 75 minutes. The fMRI tasks were initiated at 30 minutes from the start of infusion to allow plasma kisspeptin levels to reach steady state (Figure 1B).

Assays. Blood was collected to measure circulating kisspeptin, LH, follicle stimulating hormone, and testosterone levels, as previously described (30), and to confirm that baseline reproductive hormone levels were equivalent between study visits (Supplemental Table 2). Cortisol was measured on serum samples using an automated delayed 1-step immunoassay (Abbott Diagnostics) with chemiluminescent microparticle immunoassay technology. The precision of the assay was 10% or less total coefficient of variation for serum samples, with values between 83 nmol/L and 966 nmol/L. The functional sensitivity of the assay was 28 nmol/L or less, and the limit of detection was 22 nmol/L or less.

Behavioral assessments. Participants were asked to complete a set of psychometric questionnaires before their first MRI scan (Supplemental Table 1). The Patient Health Questionnaire-9 was used to screen for depression, and those scoring above the threshold for depressive disorder were excluded from the study (75). The State-Trait Anxiety Inventory, form Y, questionnaire (76) excluded anxiety traits in our cohort as all scores were within normal range (Supplemental Table 1). The Behavioral Inhibition and Activation

System Scales were used to assess sensitivity to anticipation of punishment and to reward (40). Subjective perceptions of happiness and general satisfaction with life were measured using the Subjective Happiness Scale (77) and the Satisfaction with Life Scale (78), respectively. Additionally, baseline sexual quality of life was assessed using the Sexual Quality of Life Questionnaire (43). The Sexual Desire Inventory 2 formally assessed frequency and intensity of desire in normative circumstances of both dyadic (with partner) and solitary sexual desire (42). The International Index of Erectile Function was used to screen for the 5 domains of male sexuality (desire, erectile function, intercourse satisfaction, orgasmic function, and overall satisfaction) (79), with normal baseline results in all participants (Supplemental Table 1). A second set of questionnaires was also completed by participants before and during their infusions (kisspeptin or placebo) to assess for sexual desire and emotional state in the present moment, with no differences observed between kisspeptin and placebo visits (Supplemental Tables 2 and 3). These included the Sexual Arousal and Desire Inventory to evaluate physiological, cognitive-emotional, and aversive or inhibitory components within the subjective experience of sexual desire and arousal (80) and the Profile of Mood States short form for adults, a 37-item questionnaire validated for the assessment of psychological distress using the domains of fatigue, vigor, anxiety, anger, depression, confusion, and friendliness (81).

fMRI procedure. During the MRI session, a series of anatomical and functional brain scans were performed. During the functional tasks, a mirror mounted on the head coil was used to view a screen at the rear of the scanner bore, onto which the stimuli were projected. To respond to the tasks, the participants were equipped with a custom-made, 5-button, MRI-compatible response box. In addition, a pulse oximeter and a respiratory belt were used to monitor physiological data by means of a standard data-recording system (AD Instruments PowerLab) in the control room. Kisspeptin and placebo infusions were administered via a Medrad Spectris Solaris MRI-compatible injection system controlled from a remote panel in the control room.

Olfactory task. A block design with 20 blocks was used for this task, whereby the participants received nasal delivery of Chanel No5 (concentration as per manufacturer) for 6 seconds followed by 20 seconds of odorless air as baseline. To keep the participants alert, a star appeared on the screen at random times during the task, and the participants were instructed to respond by pressing a button with their index finger on the response box. An ETT1 6-channel olfactometer (Emerging Tech Trans) was used to deliver the scent during the olfactory task. The olfactometer was connected to an odorant carrier in the MRI suite, which transported the scent to the participants via a length of Teflon tubing connected to nasal prongs worn by the participants, which allowed the olfactory stimulus to be delivered at a constant rate to the nasal passage. All participants identified Chanel No5 as pleasant and feminine at baseline (Supplemental Table 1). Participants were also asked to rate the scent during each study visit, and there were no differences in ratings between kisspeptin and placebo visits.

Facial attractiveness task. To investigate kisspeptin's effects on brain responses to varying levels of facial attractiveness on viewing opposite-sex faces, participants were presented with images of female faces from the validated Chicago Faces Database (37). Sixty faces were selected comprising of 3 groups of 20 faces rated in accordance with attractiveness (high, medium, low) by 1087 independent raters (37). Changes in brain activity in response to viewing faces of high, medium, and low attractiveness were compared between kisspeptin and placebo visits, with each participant acting as his own control. An event-related design was used in which each face was presented once for 4 seconds, followed by a jittered intertrial interval of 2 to 10 seconds. To ensure alertness, the participants were asked to rate the attractiveness of each face on a 5-point scale ranging from "very unattractive" to "very attractive" using the 5-button response box. The participants' ratings were in agreement with the independent raters, and no differences were observed between kisspeptin and placebo visits.

fMRI acquisition. Imaging data were acquired using a 3T Siemens Trio scanner with a 32-channel phased-array head coil. The anatomical images were acquired at the beginning of each scan using a T1-weighted MPRAGE pulse sequence (1-mm isotropic voxels, repetition time [TR] = 2300 ms, echo time [TE] = 2.98 ms, flip angle = 9°). For the acquisition of functional images, a multiband sequence with acceleration factor 2 was used with the following parameters for the facial attractiveness task: 3-mm voxels, TR = 1 s, TE = 30 ms, flip angle = 80°, 36 axial slices; and for the olfactory task: 2-mm voxels, TR = 1.5 s, TE = 30 ms, flip angle = 80°, 54 axial slices.

fMRI data analysis. Imaging analysis was performed using FSL. Pre-processing included motion correction, smoothing (6 mm), registration to a standard template (MNI152), and high-pass filtering (0.01 Hz). A general linear model analysis modeled the occurrence of the stimuli and included their temporal derivatives and head motion regressors as confounds. Group analyses were random effects (FLAME-1) models, with

statistical maps thresholded at $Z = 2.3$, and $P < 0.05$ (cluster corrected). Group-mean analyses including both kisspeptin and placebo visits for both tasks showed that the tasks worked effectively (Supplemental Figures 3 and 4). Group-mean analysis for the scent trials against baseline in the olfactory task showed increased activity in key parts of the human olfactory pathway (Supplemental Figure 3). Similarly, a group-mean analysis for the facial attractiveness task showed strong activation in the visual cortex, the frontal lobe, and striatal areas in the 3 attractiveness groups (high, medium, low) (Supplemental Figure 4).

A set of a priori-selected brain regions defined in standard stereotactic space using the Harvard-Oxford atlases (<https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/>) were used to extract data for ROI analyses. The ROI were selected based on evidence showing presence of kisspeptin receptors in specific areas of the human limbic brain (21) and brain regions that are crucial parts of the olfactory pathway (9). This set consisted of the following regions: accumbens, amygdala, anterior cingulate cortex, caudate, entorhinal cortex, globus pallidus, hippocampus, insula, OFC, PCC, putamen, and thalamus. Subsequently, to explore kisspeptin's effect on olfaction and reproduction further, we performed ROI analysis using olfactory and sexual arousal brain masks derived from meta-analytic data within the Neurosynth database (<http://neurosynth.org/>). A motor cortex mask was used as a control. For the facial attractiveness task, the mPFC and SFG masks were functionally defined based on the group-mean results across all participants and both conditions (kisspeptin and placebo). In addition, an ROI analysis was performed (Supplemental Figure 2) based on a priori-defined brain regions comprising areas known to express kisspeptin receptors and areas involved in sexual arousal (accumbens, amygdala, anterior cingulate cortex, caudate, globus pallidus, hippocampus, PCC, putamen, and thalamus) (21, 39).

A repeated-measures, 2 (treatment kisspeptin/placebo) by 14 (ROI) ANOVA was performed for the ROI analysis of the olfactory task. The results showed a significant effect for the ROI and treatment main effects ($P < 0.01$) but no significance in the interaction between the two. For the facial attractiveness task, a repeated-measures, 2 by 3 by 11 (ROI) ANOVA was used to assess effects between ROI, attractiveness, and treatment (kisspeptin/placebo). The results showed significant effects for the 3-way interaction ($P < 0.01$), the 2-way interaction between ROI and attractiveness ($P < 0.01$), and ROI and attractiveness as main effects ($P < 0.01$).

Statistics. Statistical analyses were performed in collaboration with a statistician. Data were normally distributed by Kolmogorov testing. Individual paired 2-tailed t tests were performed to investigate the kisspeptin effect on each individual region of interest. Pearson's correlation was used to assess correlations between brain activity and psychometric measures, with data adjusted for visit order where necessary. An α threshold of $P < 0.05$ identified statistical significance in the ROI analyses, but a reduced threshold of $P < 0.01$ was used in the correlation analysis to adjust for the number of analyses performed, in line with previous work (39). Differences between baseline and change in psychometric scores during kisspeptin compared with placebo visits were assessed using multilevel linear regression corrected for visit order.

Study approval. The study was performed in accordance with the Declaration of Helsinki. All participants gave written informed consent before inclusion in the study. The study was approved by the regional ethics committee (Riverside Research Ethics Committee, London, United Kingdom, REC 17/LO/1504).

Author contributions

LY, LD, MBW, EAR, ANC, and WSD conceived the study and designed the protocol. LY, LD, EGAM, DZ, MS, and JKP collected the data. LY, LD, MBW, AA, PAB, and ANC analyzed the data. LY, LD, MBW, BMO, ANC, and WSD prepared the manuscript. ANC and WSD supervised all aspects of the work.

Acknowledgments

This study presents independent research funded by the Medical Research Council (MRC) and supported by the NIHR Clinical Research Facility and NIHR Biomedical Research Centre at Imperial College Healthcare NHS Trust. LY is funded by an MRC Clinical Training Fellowship (MR/R000484/1). EGAM was supported by an Imperial Healthcare NHS Charity Fellowship, and DZ and MS were supported by the NIHR Academic Foundation Programme during the study. AA is supported by a NIHR Clinician Scientist fellowship. BMO is funded by a Sir Henry Dale Fellowship (105545/Z/14/Z) jointly funded by the Wellcome Trust and the Royal Society. ANC is supported by the NHS. WSD is funded by an NIHR Professorship (NIHR RP-2014-05-001). The views expressed are those of the authors and not necessarily those of the MRC, NHS, NIHR, or Department of Health.

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ORIGINAL ARTICLE

The effects of kisspeptin on β -cell function, serum metabolites and appetite in humans

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Aims: To investigate the effect of kisspeptin on glucose-stimulated insulin secretion and appetite in humans.

Materials and methods: In 15 healthy men (age: 25.2 ± 1.1 years; BMI: $22.3 \pm 0.5 \text{ kg m}^{-2}$), we compared the effects of $1 \text{ nmol kg}^{-1} \text{ h}^{-1}$ kisspeptin versus vehicle administration on glucose-stimulated insulin secretion, metabolites, gut hormones, appetite and food intake. In addition,

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Diabetes Obes Metab. 2018;20:2800–2810.

Funding information

Biotechnology and Biological Sciences Research Council; Diabetes UK; FP7-HEALTH- 2009- 241592 EuroCHIP grant; Integrative Mammalian Biology Capacity Building Award; Medical Research Council; National Institute for Health Research; Wellcome Trust

we assessed the effect of kisspeptin on glucose-stimulated insulin secretion in vitro in human pancreatic islets and a human β -cell line (EndoC- β H1 cells).

Results: Kisspeptin administration to healthy men enhanced insulin secretion following an intravenous glucose load, and modulated serum metabolites. In keeping with this, kisspeptin increased glucose-stimulated insulin secretion from human islets and a human pancreatic cell line in vitro. In addition, kisspeptin administration did not alter gut hormones, appetite or food intake in healthy men.

Conclusions: Collectively, these data demonstrate for the first time a beneficial role for kisspeptin in insulin secretion in humans in vivo. This has important implications for our understanding of the links between reproduction and metabolism in humans, as well as for the ongoing translational development of kisspeptin-based therapies for reproductive and potentially metabolic conditions.

KEYWORDS

appetite control, beta cell function, glucose metabolism, incretins, insulin secretion, islets

1 | INTRODUCTION

Metabolism and reproduction are fundamental aspects of mammalian physiology which are intricately linked. However, our understanding of the hormonal links between these two biological systems is limited. Recent evidence in animals suggests that the recently discovered hormone kisspeptin may link reproduction and metabolism; however, until now human in vivo studies have not been performed.

Kisspeptin sits at the apex of the hypothalamo-pituitary-gonadal axis, controlling downstream reproductive hormone secretion. Kisspeptin has pivotal roles in fertility,¹ which are being utilised by kisspeptin-based therapies now in development for common reproductive disorders.^{2–5} Kisspeptin (*kiss1*) and its receptor (*kiss1r*) are expressed in the hypothalamus, pancreatic β -cells, liver and adipose tissue,^{6–9} suggesting roles in key autocrine and paracrine metabolic processes. Kisspeptin has recently been reported to increase glucose-stimulated insulin secretion (GSIS) in rats¹⁰ and monkeys,¹¹ and this is supported by in vitro islet studies which demonstrate enhancement of GSIS by kisspeptin.^{8,10,12,13} Interestingly, it has also been reported that kisspeptin can inhibit GSIS at lower kisspeptin concentrations¹³ and low glucose concentrations,¹⁴ but stimulate GSIS at high kisspeptin concentrations.¹³

There are neuroanatomical and functional connections between kisspeptin and important hypothalamic appetite-regulating neuropeptides.^{15–17} Intracerebroventricular administration of kisspeptin has been reported to alter food intake in mice¹⁸ and chicks,¹⁹ while other studies report no effect of kisspeptin administration on appetite in rats.²⁰ In addition, impaired kisspeptin signalling disrupts metabolism and promotes glucose intolerance and obesity in mice.²¹

Thus, data in animals strongly suggests that kisspeptin plays a role in metabolism, but the effects of kisspeptin on metabolic parameters in vivo in humans are currently unknown. There is therefore an important clinical need to elucidate the metabolic effects of kisspeptin in humans, to further our understanding of the physiology of human metabolism, as well as to inform the ongoing development of kisspeptin-based therapies.

In this study, we investigate the effects of kisspeptin on β -cell function, metabolites and appetite in humans. We demonstrate that in

humans, administration of kisspeptin stimulates GSIS in vivo and in vitro, and significantly modulates the concentrations of metabolites, some of which are known to be associated with insulin secretion. Furthermore, kisspeptin administration did not affect appetite or food intake. These data provide the first human in vivo insights into the metabolic actions of kisspeptin and have significant translational implications relating to glucose homeostasis.

2 | MATERIALS AND METHODS**2.1 | Human studies****2.1.1 | Study participants**

This study was reviewed and approved by the West London Research Ethics Committee (16/LO/0391), and was performed in accordance with the Declaration of Helsinki. Healthy men (aged 18–40 years) were recruited by online and print advertisements. Written informed consent was obtained from each participant prior to study enrolment. Exclusion criteria included body mass index (BMI) < 18.5 or > 25 kg m⁻², a history of medical and psychological conditions, use of prescription, recreational or investigational drugs within the preceding 2 months, blood donation within 3 months of study participation, ingestion or inhalation of nicotine-containing substances, alcoholism, and history of cancer.

Participants were instructed to abstain from strenuous exercise, alcohol and caffeine for 24 hours preceding each study visit. Each participant was instructed to choose a meal and eat that same meal at 8 pm on the night preceding each study visit, fast overnight, and attend the study visit fasted. Each participant underwent two intravenous glucose tolerance tests (IVGTTs, one with kisspeptin and one with vehicle administration) and/or two mixed meal tolerance tests (MMTTs, one with kisspeptin and one with vehicle administration).

2.1.2 | Infusions

Infusion order was randomized using a random number generator, and participants were blinded as to the identity of the infusion. Kisspeptin infusions were prepared by dissolving kisspeptin-54 (Bachem, St Helens, uk) in 1 mL of 0.9% NaCl (Braun, Sheffield, UK) then adding the

kisspeptin solution to 49 mL Gelofusine (Braun). Kisspeptin was infused at a rate of $1 \text{ nmol kg}^{-1} \text{ h}^{-1}$, a dose previously established to be bioactive in humans.⁵ Vehicle infusions consisted of Gelofusine administered at the equivalent rate to the kisspeptin infusion for each participant.

2.1.3 | IVGTT

On arrival at the Clinical Research Facility (CRF), and after a period of acclimatisation, two intravenous cannulae (one in each antecubital fossa) were inserted (one for blood sampling and the other for intravenous infusion administration). Following baseline sampling, kisspeptin or vehicle infusion was started at $T = 0$ minutes and infused until $T = 225$ minutes. Next, 0.3 g kg^{-1} of 20% dextrose (Hameln, Gloucester, UK) was administered intravenously over 120 seconds starting from $T = 45$ minutes (i.e. when kisspeptin levels had reached a steady state, Figure 1A,B). To obtain the glucose and insulin values required for calculation of the acute insulin response to glucose (AIRg) and minimal model insulin sensitivity index (S_i), an established frequent sampling protocol²² was used (Figure 1A). AIRg was calculated as the incremental AUC (using the trapezoid rule²³) of insulin from $T = 45$ minutes to $T = 55$ minutes (i.e. 0–10 minutes post-glucose load). S_i was determined using the minimal model (MLAB software)²⁴ and the disposition index (IVGTT-DI) was calculated as the product of AIRg and S_i .²² Blood was taken for measurement of glucose, insulin, kisspeptin, luteinising hormone (LH), testosterone, glucagon-like peptide-1 (GLP-1), peptide-YY (PYY), glucagon and cortisol.

2.1.4 | MMTT

A taste test was performed prior to the MMTT study visits to determine the study meal (spaghetti bolognese [$125 \text{ kcal } 100 \text{ g}^{-1}$] or mushroom risotto [$124 \text{ kcal } 100 \text{ g}^{-1}$]; both from Waitrose, Bracknell, UK) for each participant. These meal options were selected as they provided similar kcal weight⁻¹. Following acclimatisation and baseline sampling, kisspeptin or vehicle infusion was started at $T = 0$ minutes and continued until $T = 120$ minutes.

Visual analogue scales, to measure participants' self-reported hunger, were performed at $T = -30$ minutes, $T = 30$ minutes and $T = 75$ minutes. Next, 45 minutes into the infusion (i.e. when kisspeptin levels had reached a steady state), an ad libitum meal was given to participants. They were instructed to eat until comfortably full; all clocks, electronic devices and reading material were taken away while the participants were eating, and researchers left the room (to minimize distractions which are known to influence meal size and duration). Blood was taken for the measurement of glucose, insulin, kisspeptin, LH and testosterone.

Calculations were made using established methods as follows: MMTT-ISI (MMTT insulin secretion index) as the ratio of AUC insulin to AUC glucose, MMTT-IS (MMTT insulin sensitivity index) using the Matsuda index (with $T = 45$ minutes values as baseline, and mean insulin and glucose values from $T = 65$ –120 minutes used in the equation), and MMTT-DI (MMTT disposition index) as the product of MMTT-ISI and MMTT-IS.²⁵

2.1.5 | Serum metabolite identification and analyses

Blood samples for metabolic profiling were taken pre-infusion ($T = -15$ minutes) and at steady state prior to intravenous glucose or

meal ingestion ($T = 45$ minutes). Serum sample handling (sorting and formatting) was performed as previously described,²⁶ and $50 \mu\text{L}$ aliquots were taken from each sample for metabolite analyses. Protein was removed from the samples prior to analysis by addition of organic solvent, mixing and centrifugation, yielding a homogenous supernatant (method-specific details are included in the supporting information for this article). The prepared samples were subjected to small molecule and lipid analyses by ultra-performance liquid chromatography mass spectrometry (UPLC-MS). Reversed-phase chromatography tailored for complex lipid retention and separation was used to profile the lipid species of each sample, while hydrophilic interaction liquid chromatography (HILIC) was used to retain and separate polar metabolites.²⁶ High resolution orthogonal acceleration time-of-flight mass spectrometry (oaTOF-MS) operating in the positive ion mode was used for both assays.

All UPLC-MS analyses were performed on Acquity UPLC instruments, coupled to Xevo G2-S TOF mass spectrometers (Waters Corp., Manchester, UK) via a Z-spray electrospray ionisation (ESI) source. Further details of the analytical methods used can be found in the supporting information for this article. Feature extraction and retention time alignment were performed in Progenesis Q1 (Waters Corp., Milford, MA). In-house scripts (Python software) were developed for the elimination of potential run-order effects and noise filtering. Linear mixed effect models were generated using the lmer4 R software package²⁷ according to the formula: $\text{model} \leftarrow \text{Feature} \sim \text{Time} * \text{Class} + (1|\text{SubjectID}) + (1|\text{Challenge})$. A model was generated for each feature including fixed effects for interaction between class (kisspeptin or vehicle alone) and time ($T = -15$ minute and $T = 45$ minute), allowing for both participant and challenge-specific random effects (owing to the presence of multiple challenges per participant). Features showing significant differences between the two timepoints for vehicle and/or kisspeptin classes were identified by false discovery rate correction of the appropriate model estimates using the locfdr package.²⁸ Subsequently, assignment of significant features in the kisspeptin class alone (FDR $\alpha = 0.05$) was performed. Where significant features were found to be adduct or fragment ions, the full spectrum (including the protonated molecule) was deduced and used in molecular assignment. Chemical identity was assigned by matching accurate mass and tandem mass spectrometry (MS/MS) fragmentation (of the protonated molecule) measurements to reference spectra using LIPID MAPS online tools (for lipid species)²⁹ or an in-house database constructed from analysis of authentic reference materials. Where authentic reference materials were commercially available, they were used to generate definitive molecular identification by direct matching of chromatographic and spectral qualities (including accurate mass, MS/MS spectra, and isotopic distribution) to those observed in the profiling data and subsequent targeted MS/MS experiments.

2.1.6 | Biochemical analyses

Plasma kisspeptin and gut hormone levels were measured using established in-house radioimmunoassays (kisspeptin intra-assay CV 8.3% and inter-assay CV 10.2%, GLP-1 intra-assay and inter-assay CV: $\leq 10\%$, PYY intra-assay and inter-assay CV: $\leq 10\%$, glucagon intra-assay and inter-assay CV: $\leq 10\%$).^{5,22,30} Serum insulin, plasma glucose, serum

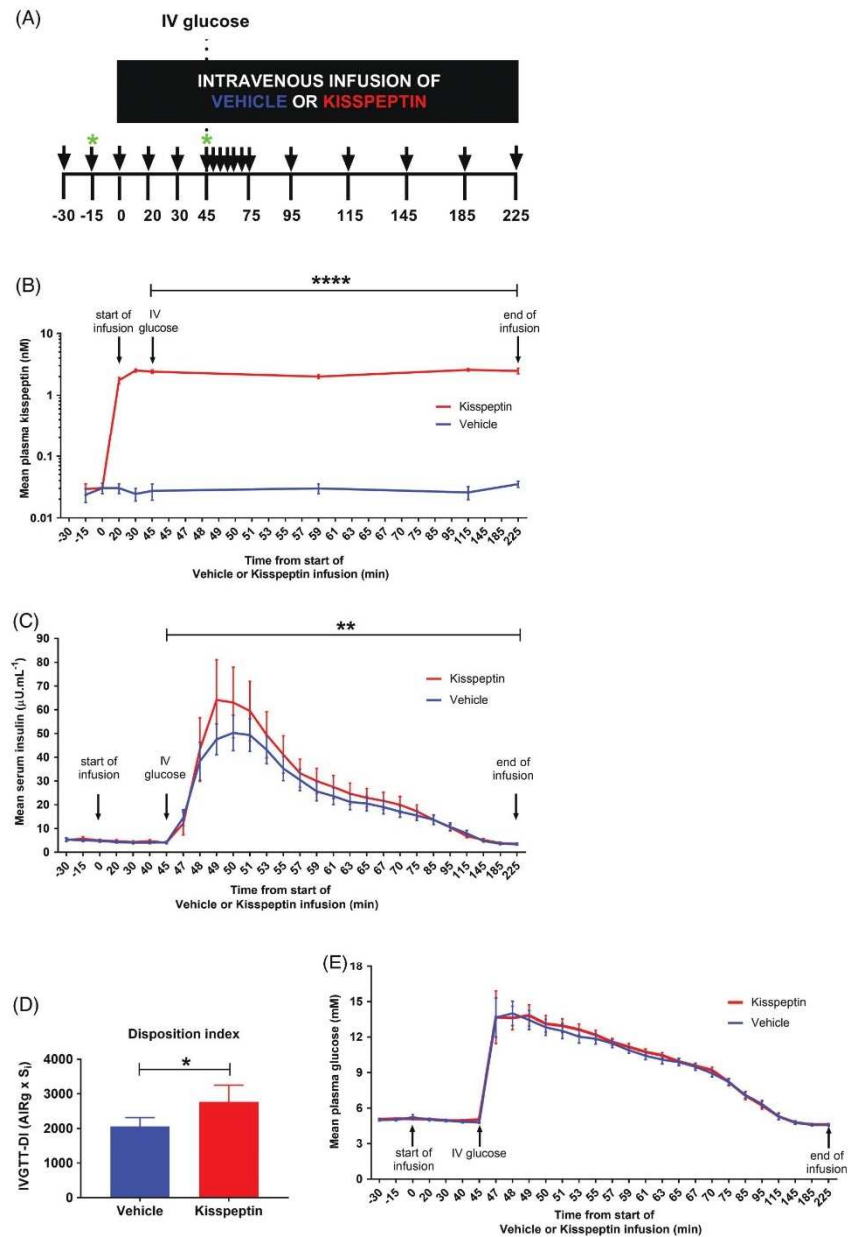


FIGURE 1 Kisspeptin administration enhances β -cell function during IVGTT in humans. A, Following an overnight fast, 15 healthy men (age: 25.2 ± 1.1 years; BMI: $22.3 \pm 0.5 \text{ kg M}^{-2}$) were administered $1 \text{ nmol kg}^{-1} \text{ hr}^{-1}$ kisspeptin or rate-matched vehicle for 225 minutes, in random order. At T = 45 minutes, 0.3 g kg^{-1} of 20% dextrose was infused intravenously over 2 minutes. Blood sampling was performed regularly (black arrows), with samples for metabolic profiling collected at T = -15 minutes and T = 45 minutes (green stars). B, Mean plasma kisspeptin levels during IVGTT were higher during kisspeptin infusion compared with vehicle administration as expected; **** $P < 0.0001$ kisspeptin vs. vehicle using GEE. C, Mean serum insulin levels during IVGTT were higher during kisspeptin infusion compared with vehicle administration; ** $P = 0.01$ kisspeptin vs. vehicle (multi-level linear regression). D, Mean IVGTT-DI was higher during kisspeptin administration (red bar) compared with vehicle administration (blue bar); * $P < 0.05$ kisspeptin vs. vehicle (paired t-test). E, Mean plasma glucose levels during IVGTT were similar during kisspeptin and vehicle administration; $P = 0.64$ kisspeptin vs. vehicle (multi-level linear regression). Data are presented as mean $\pm$ SEM; $n = 15$ per group

LH, serum testosterone and serum cortisol were measured in the Clinical Chemistry Laboratory of Imperial College Healthcare NHS Trust on the automated Abbott Architect® platform. Chemiluminescent immunoassays were used to measure serum insulin (intra-assay and inter-assay CV: $\leq 7\%$), serum LH (intra-assay and inter-assay CV: $\leq 5\%$), serum testosterone (intra-assay and inter-assay CV: $\leq 8\%$) and serum cortisol (intra-assay and inter-assay CV: $\leq 10\%$). Plasma glucose was measured using a colorimetric hexokinase assay (intra-assay and inter-assay CV: $\leq 2\%$).

2.2 | In vitro studies

2.2.1 | Human islet cell culture

Preparations from six different donors (Table S1 in File S1) were used to perform insulin secretion experiments. Islets (10/well) were incubated in triplicate for each condition in a 12-well non-treated cell culture plate. Insulin secretion assays were performed in Krebs-Ringer-Hepes-Bicarbonate (KRHB) buffer (10 mM Hepes, 2 mM NaHCO_3 , 140 mM NaCl, 3.6 mM KCl, 0.5 mM MgSO_4 , 0.5 mM NaH_2PO_4 , 1.5 mM CaCl_2 supplemented with 0.1% BSA) saturated with 95% $\text{O}_2/5\%$ CO_2 and adjusted to pH 7.4.³¹ Islets were pre-incubated in a 37 °C water bath under agitation for 1 hour in 3 mM glucose KRHB prior to the secretion assay (for 30 minutes) in KRHB, 3 mM or 17 mM glucose, in the presence of different concentrations of kisspeptin-54 (0, 2.7 or 1000 nM). The 2.7 nM dose was selected as this is similar to the plasma kisspeptin levels produced by 1 nmol.kg⁻¹ h⁻¹ kisspeptin administration in our in vivo study (i.e. 2-3 nM during kisspeptin steady state – see Figure 1B). The 1000 nM dose was selected as this was similar to the dose of kisspeptin used in other in vitro studies.^{8,10,13} The supernatant was collected and the islets were lysed in 1 mL of acidified ethanol (75% ethanol, 23.5% H_2O , 1.5% 1 M HCl, 0.1% Triton) and sonicated two times for 10 seconds, to extract total islet insulin content. Insulin concentration was measured using an ultrasensitive HTRF kit (Cisbio Bioassays, Codolet, France), and secreted insulin was normalized as percentage of total insulin content.

2.2.2 | Human β -cell line (EndoC- β H1 cells)

EndoC- β H1 cells were seeded onto ECM-coated 48-well plates at 250×10^3 cells per well. Four days after seeding, cells were incubated overnight in a glucose starving medium (glucose-free DMEM-2% albumin from bovine serum fraction V, 50 μL 2-mercaptoethanol, 10 mM nicotinamide, 5.5 $\mu\text{g mL}^{-1}$ transferrin, 6.7 ng mL⁻¹ sodium selenite and penicillin/streptomycin) with glucose added to give a final concentration of 2.8 mM glucose. The next morning cells were incubated for 1 hour with Krebs Ringer solution (0.2% BSA, 25% solution I, 25% solution II, 25% solution III, 10 mM Hepes) supplemented with 15 mM glucose. EndoC- β H1 cells were then incubated in the presence of different concentrations of kisspeptin-54 (0, 100 or 1000 nM). These doses are similar to those used in published in vitro studies.^{8,10,13} The cells were incubated in triplicate for each condition. After incubation for 60 minutes, the supernatant was collected, placed onto ice and centrifuged for 5 minutes at 3000 rpm at 4 °C. The supernatant was then transferred into a fresh tube. Cells were lysed in 50 μL of cell lysis solution (TETG: 20 mM Tris PH 8.0, 1% Triton X-100, 10% glycerol, 137 mM NaCl, 2 mM EGTA). The lysate was

then removed and placed onto ice, and centrifuged at 3000 rpm for 5 minutes at 4 °C. Insulin concentration was measured using an ultrasensitive HTRF kit (Cisbio Bioassays), and secreted insulin was normalized as percentage of total insulin content.

2.3 | Statistical methods

Using STATA, an a priori power calculation was performed using a dataset of IVGTTs performed in 99 healthy men aged 18-40 years, provided by I.F.G. Using this dataset, a study consisting of IVGTTs performed in 15 healthy men would have 80% power to detect a 25% difference in insulin secretion.

Statistical analyses were performed with the assistance of a statistician (P.Ba). Unless otherwise stated, statistical analysis was performed using Prism (GraphPad, CA) and data are presented as mean $\pm$ SEM. Paired t-tests were performed on parametric data and Wilcoxon matched-pairs signed rank tests were performed on non-parametric data. Two-way ANOVA with Dunnett's multiple comparison tests was performed for comparison of >2 groups of parametric data, while a Friedman test with Dunn's multiple comparison tests was performed for comparison of >2 groups of non-parametric data. Multi-level linear regression modelling was performed on insulin and glucose curves. The generalized estimating equation (GEE) was performed on other non-independent longitudinal data using STATA (Statacorp, TX). Statistical significance was set at $P < 0.05$.

3 | RESULTS

3.1 | Elevated circulating kisspeptin enhances insulin secretion during intravenous glucose challenges in healthy men

To establish the effect of kisspeptin administration on GSIS in vivo in humans, 1 nmol kg⁻¹ h⁻¹ kisspeptin infusion (a dose of kisspeptin that has previously been shown to be bioactive in humans⁵), or rate-matched vehicle infusion, was administered to 15 healthy young men in a randomized blinded two-way crossover study. Using a well-established frequently sampled IVGTT protocol²² (Figure 1A), a 0.3 g kg⁻¹ intravenous glucose load was administered at T = 45 minutes, when circulating kisspeptin levels had reached steady state (Figure 1B). In response to the intravenous glucose load, kisspeptin administration significantly enhanced GSIS compared with vehicle (Figure 1C). Two-level linear regression modelling confirmed that mean post-glucose load insulin levels were 4.1 $\mu\text{U mL}^{-1}$ higher (95% CI: 0.9-7.3; $p = 0.01$) during kisspeptin compared with vehicle administration. The insulin sensitivity index (S_i) (S_i : kisspeptin $8.11 \pm 0.98 \text{ min}^{-1} \text{ mU}^{-1} \text{ L} \cdot 10^4$ vs. vehicle $6.85 \pm 0.89 \text{ min}^{-1} \text{ mU}^{-1} \text{ L} \cdot 10^4$, $p = 0.1228$) was similar between groups.

Furthermore, kisspeptin elicited a significantly higher disposition index (IVGTT-DI) compared with vehicle (IVGTT-DI: kisspeptin 2768 ± 484 units vs. vehicle 2061 ± 255 units, $p < 0.05$, Figure 1D). The disposition index is a well-validated method for assessing β -cell function³² as it is comprised of measures of insulin secretion, insulin

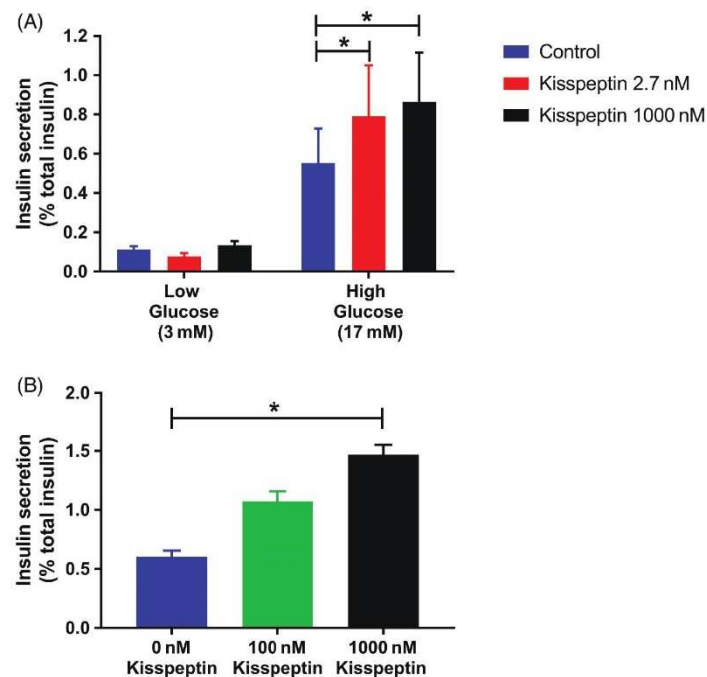


FIGURE 2 Kisspeptin enhances GSIS in cultured human donor islets and a human pancreatic β -cell line. A, Insulin secretion was measured in human islet preparations ($n = 6$ donors) at low (3 mM) and high (17 mM) concentrations of glucose in the presence of increasing concentrations of kisspeptin (0 nM [blue bars], 2.7 nM [red bars] or 1000 nM [black bars]). Insulin secretion was normalized to percentage of total secretion; * $p < 0.05$ (Friedman test with Dunn's multiple comparison tests). B, Insulin secretion in cultured EndoC- β H1 cells ($n = 3$ experiments) was measured at high glucose concentrations (15 mM) in the presence of increasing amounts of kisspeptin (0 nM [blue bars], 100 nM [green bars] or 1000 nM [black bars]). Insulin secretion was normalized to percentage of total secretion; * $p < 0.05$ (two-way ANOVA with Dunnett's multiple comparison tests). Data presented as mean $\pm$ SEM

sensitivity and prevailing glucose concentrations (which were similar between groups; Figure 1E).

Kisspeptin administration resulted in elevated circulating kisspeptin levels (Figure 1B), which, as expected, resulted in elevated LH levels, confirming peptide bioactivity (Figure S1A). Consistent with previous studies,^{5,33} testosterone levels did not rise during the time-period of kisspeptin administration (Figure S1B).

Kisspeptin administration did not alter the circulating levels of endogenous insulin secretagogues GLP-1 (Figure S1C), PYY (Figure S1D) and glucagon during IVGTT (Figure S1E). Additionally, kisspeptin administration did not affect circulating levels of cortisol, a counter-regulatory hormone (Figure S1F).

3.2 | Kisspeptin dose-dependently stimulates GSIS in vitro

To assess the effect of kisspeptin on GSIS in vitro, human islets from six donors were incubated with 3 and 17 mM glucose with a range of concentrations of kisspeptin (0, 2.7 and 1000 nM kisspeptin). In donor human islets, kisspeptin enhanced insulin secretion (Figures 2A and S2), providing key mechanistic evidence that kisspeptin can stimulate GSIS by direct action on β -cells. Crucially, kisspeptin stimulated human islet insulin

secretion (in the presence of elevated glucose levels, Figure 2A) at similar kisspeptin concentrations to the circulating kisspeptin levels obtained in our human in vivo IVGTT studies above (i.e. 2–3 nM, Figure 1B). Incubation with kisspeptin increased the insulin stimulation index (insulin stimulation index: 2.7 nM kisspeptin 11.08 ± 3.39 vs. 0 nM 5.09 ± 1.18 , $p < 0.01$; 1000 nM 6.83 ± 1.84 vs. 0 nM 5.09 ± 1.18 , $p = 0.0866$), but did not affect the insulin content of the donor islet preparations (Figure S3).

To further evaluate the effect of kisspeptin on GSIS in vitro, EndoC- β H1 cells (a validated human β -cell line, which retains many of the features of human pancreatic β -cells)^{34,35} were incubated with 15 mM glucose and kisspeptin concentrations, which have been used previously to investigate the effects of kisspeptin on GSIS in vitro (0, 100 and 1000 nM kisspeptin).^{8,10,13} Similar to the donor human islets, kisspeptin increased insulin secretion from EndoC- β H1 cells in a dose-dependent manner (Figure 2B).

3.3 | Kisspeptin alters the pattern of specific serum metabolites in humans

To provide further insights into the metabolic effects of kisspeptin, blood samples from 15 volunteers during 30 $1 \text{ nmol kg}^{-1} \text{ h}^{-1}$

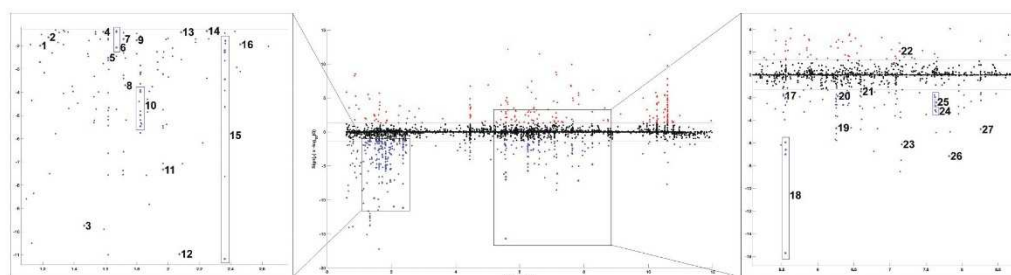


FIGURE 3 Kisspeptin modulates the metabolic profile in healthy men. Manhattan plot of the 5200 lipid species detected (in serum samples from 15 healthy male volunteers) by UPLC-MS. 392 features showing a change over time significantly associated with kisspeptin administration are coloured red (increasing) or blue (decreasing). Statistical significance was determined based on a Q value threshold of 5%, where Q represents the local FDR-corrected value of the appropriate linear mixed effect model estimates. Features successfully annotated are indicated on the plot as follows: 1, LPI(20:4); 2, LPC(0:0/14:0); 3, LPI(18:1); 4, LPE(0:0/18:2); 5, LPC(18:2/0:0); 6, LPE(18:2/0:0); 7, LPC(0:0/16:0); 8, CAR(20:3); 9, LPC(20:3/0:0); 10, LPC(16:0/0:0); 11, LPC(18:1/0:0); 12, Sphinganine(d18:0); 13, LPC(20:2/0:0); 14, LPC(0:0/18:0); 15, LPC(18:0/0:0); 16, LPC(20:1/0:0); 17, PC(18:2/18:2); 18, SM(d18:1/16:0); 19, PC(16:0/16:0); 20, SM(d18:1/18:0); 21, PC(18:0/18:2); 22, SM(d18:2/24:1); 23, SM(d18:2/22:0); 24, SM(d18:1/24:1); 25, SM(d18:1/22:0); 26, SM(d18:2/24:0); 27, SM(d18:1/24:0). Where LPI: lysophosphatidylinositol; LPC: lysophosphocholine; PC: phosphocholine; SM: sphingomyelin; LPE: lysophosphatidylethanolamine; CAR: Fatty acyl carnitine (see also Table S4)

kisspeptin infusions and 30 vehicle infusions were taken at $T = -15$ minutes (i.e. pre-infusion) and at $T = 45$ minutes (i.e. when kisspeptin had reached steady state but prior to the glucose load) (Figure 1A), and serum was analysed using UPLC-MS. Linear mixed effect modelling²⁷ was used to identify patterns of serum metabolites, which changed significantly between baseline ($T = -15$ minutes) and steady state ($T = 45$ minutes) during kisspeptin but not vehicle administration. The profiles of numerous small molecules (Figure S4) and lipid species (Figure 3) were altered, including lysophosphatidylcholine (LPC 18:2), which was reduced by kisspeptin administration and has previously been shown to be negatively correlated with insulin secretion.³⁶ All annotated metabolites that significantly changed during kisspeptin administration (but not vehicle administration) have been collated in Table S4.

3.4 | Kisspeptin does not affect appetite or food intake in humans

To assess the effect of kisspeptin on appetite and food intake in humans, $1 \text{ nmol kg}^{-1} \text{ h}^{-1}$ kisspeptin infusion, or rate-matched vehicle infusion, was administered to 15 healthy young men. Participants were asked to record hunger and appetite on a visual analogue scale (VAS, range 0–10 cm) at several timepoints during the infusion and were given an ad libitum meal at $T = 45$ minutes (i.e. when kisspeptin levels were at steady state) (Figure 4A). Self-reported hunger was not significantly different during kisspeptin or vehicle administration (increase in hunger VAS scores from $T = -30$ minutes (baseline) to $T = 30$ minutes (i.e. 30 minutes after the start of the infusion): kisspeptin $1.88 \pm 0.63 \text{ cm}$ vs. vehicle $1.69 \pm 0.40 \text{ cm}$, $p = 0.8120$) (Figure 4B). Hunger VAS scores from $T = 30$ minutes (pre-meal) to $T = 75$ minutes (post-meal) decreased similarly during kisspeptin and vehicle administration (pre-meal to post-meal change in VAS: kisspeptin $-6.02 \pm 0.56 \text{ cm}$ vs. vehicle $-5.6 \pm 0.49 \text{ cm}$, $p = 0.5183$).

Furthermore, there was no difference in the mean kilocalories (kcal) consumed during the ad libitum meal during kisspeptin and

vehicle administration (kisspeptin $844.7 \pm 160.9 \text{ kcal}$ vs. vehicle $834.4 \pm 174.8 \text{ kcal}$, $p = 0.7178$) (Figure 4C).

As expected, following ad libitum meal ingestion, mean glucose levels achieved were lower than during IVGTT post-glucose load (mean glucose level: IVGTT $9.72 \pm 0.49 \text{ mM}$ vs. MMTT $6.62 \pm 0.20 \text{ mM}$, $p < 0.05$), with no associated effect of kisspeptin administration on insulin levels (Figure S5A), glucose levels (Figure S5B) or MMTT-DI (kisspeptin $696.3 \pm 159.1 \text{ units}$ vs. vehicle $671.9 \pm 145.6 \text{ units}$, $p = 0.6286$).

During kisspeptin administration, plasma kisspeptin levels were elevated (Figure 4D), which resulted in elevated LH levels (Figure S5C) and no significant change in testosterone (Figure S5D), similar to those from IVGTTs.

4 | DISCUSSION

This is the first study investigating the effect of kisspeptin on β -cell function and appetite in humans in vivo. We demonstrate that the reproductive hormone kisspeptin enhances GSIS in humans in vivo without affecting insulin sensitivity. Our in vitro data show a direct effect of kisspeptin on β -cells, which are known to possess abundant kisspeptin receptors.⁸ Furthermore, kisspeptin modulates serum metabolites in humans, but does not influence appetite or food intake. These data indicate that kisspeptin plays a role in glucose homeostasis in humans, and therefore is a hormonal mediator linking reproductive and metabolic systems.

Kisspeptin administration increased insulin secretion and disposition index during IVGTT but not during MMTT. The glucose concentrations were more markedly elevated (as expected) during the IVGTT (mean peak glucose 14.01 mM , Figure 1E) compared to the MMTT (mean peak glucose 7.48 mM , Figure S5B). Therefore, this suggests that kisspeptin increases insulin release only when ambient glucose concentrations are high in humans in vivo. In keeping with this, our data show that in human islets in vitro, kisspeptin increases insulin

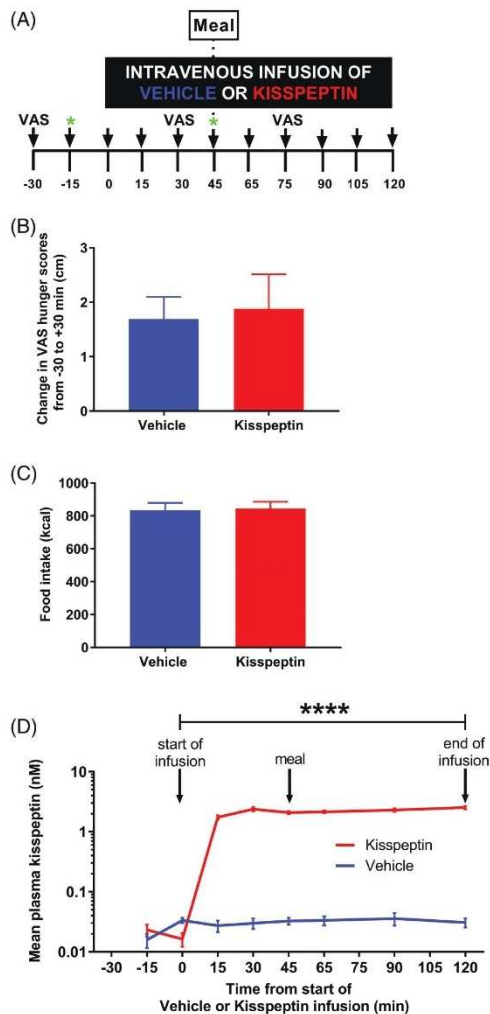


FIGURE 4 Kisspeptin administration does not affect appetite or food intake in healthy men. A, Following an overnight fast, 15 healthy men (age: 25.2 ± 1.1 years; BMI: $22.3 \pm 0.5 \text{ kg m}^{-2}$) were administered $1 \text{ nmol kg}^{-1} \text{ h}^{-1}$ kisspeptin or rate-matched vehicle for 120 minutes, in random order. At T = 45 minutes, participants were given an ad libitum meal (which was eaten over a maximum of 20 minutes). Blood sampling was performed regularly (black arrows), with samples for metabolic profiling collected at T = -15 minutes and T = 45 minutes (green stars). Visual analogue scores (VAS) for volunteer-reported hunger were recorded at T = -30 minutes, T = 30 minutes and T = 75 minutes. B, Mean change in pre-meal volunteer-reported hunger scores from T = -30 minutes to T = 30 minutes, as measured with VAS, were similar during kisspeptin (red bar) and vehicle (blue bar) administration; $p = 0.8120$ kisspeptin vs. vehicle (paired t-test). C, Mean number of kcal ingested was similar during kisspeptin (red bar) and vehicle (blue bar) administration; $p = 0.7178$ kisspeptin vs. vehicle (paired t-test). D, During MMTT, mean plasma kisspeptin levels were higher during kisspeptin compared with vehicle administration as expected; **** $p < 0.0001$ kisspeptin vs. vehicle (GEE). Data are presented as mean $\pm$ SEM; $n = 15$ per group

secretion at 17 mM but not at 3 mM glucose (Figure 2A). This is also consistent with previous data in human islets in vitro, which show that kisspeptin stimulates insulin release at higher ambient glucose concentrations (20 vs. 3 mM).^{8,12}

However, there are differing reports of the effects of kisspeptin on insulin secretion from animal islets. Kisspeptin, at doses ranging from 0.1–1000 nM, has been reported to increase GSIS in mouse, rat and pig islets, when the prevailing glucose concentrations were $\geq 10 \text{ mM}$.^{8,10,12,37} In contrast, other investigators have reported that kisspeptin, at doses ranging from 0.1–1000 nM, inhibits GSIS in a dose-dependent manner in mouse and rat islets/perfused pancreata.^{13,14,38} The lack of consensus in the literature may be due to methodological differences in the experiments and/or species differences, which are often observed between animal and human islet preparations.³⁹ Our human data in a range of kisspeptin doses in vitro and at $1 \text{ nmol kg}^{-1} \text{ h}^{-1}$ in vivo in the current study do, however, demonstrate positive effects of kisspeptin on GSIS. Nevertheless, given that only one concentration of kisspeptin was used in our in vivo study, further studies are required to determine the effects of kisspeptin on GSIS in vivo across a dose spectrum.

It is interesting to note that the increase in post-load IVGTT insulin concentrations produced by kisspeptin administration was not accompanied by a change in glucose levels. This could suggest some loss of sensitivity to insulin. However, S_i , as measured with the minimal model, was not reduced. Additionally, fasting insulin concentrations, which rise with reductions in S_i ,⁴⁰ were unaffected by kisspeptin. Another explanation for the maintenance of glucose levels could be counter-regulatory cortisol secretion. However, we measured cortisol levels in the IVGTT samples and there was no significant difference in cortisol during kisspeptin administration compared to vehicle (Figure S1F). Overall, it appears that although kisspeptin enhanced insulin secretion, in healthy individuals this did not result in altered glucose levels, but this would be interesting to investigate in patients with abnormal glucose homeostasis such as in diabetes.

Testosterone has also been shown to increase insulin secretion from isolated islets.⁴¹ However, the effects of kisspeptin administration on metabolism during our study were not confounded by altered circulating testosterone levels as serum testosterone did not rise during the time-period of this study, because more prolonged kisspeptin administration is required to produce elevations in testosterone levels.³³

The gut hormones, GLP-1, PYY and glucagon have key roles in glucose homeostasis. Additionally, in rodent studies, GLP-1 has been shown to alter hypothalamic kisspeptin expression and neuronal activity,^{42,43} and in mice glucagon stimulates hepatic kisspeptin production to alter GSIS.¹³ In our study, there was no difference in circulating GLP-1, PYY or glucagon levels following intravenous glucose during kisspeptin administration compared to vehicle (Figure S1C–E). This is consistent with previous data which show that intravenous glucose administration alone does not activate the incretin response mediated by gut hormones.⁴⁴ Furthermore, the above studies suggest that GLP-1 and glucagon may act upstream of kisspeptin, whereas in our study we directly administered kisspeptin, which may act downstream of GLP-1 and glucagon.

The participants were fasted during the IVGTTs, and fasting has been shown to reduce hypothalamic kisspeptin expression.⁴⁵ However, as shown in Figure 1B, kisspeptin administration significantly raised plasma kisspeptin levels, and therefore would be sufficient to overcome the effect of suppression of endogenous kisspeptin by fasting. Furthermore, kisspeptin administration has been shown to increase GSIS in both fasted and fed monkeys.¹¹

Metabonomics, the identification and analysis of metabolites in biological fluids, is an emerging field of study which provides a non-biased methodology to identify novel pathways to guide further research. To provide further insights into the metabolic effects of kisspeptin, we compared the distribution of metabolites in serum samples taken pre-kisspeptin administration ($T = -15$ minutes) to those taken when plasma kisspeptin levels had reached steady state ($T = 45$ minutes) and equivalent vehicle timepoints. Importantly, samples were collected before the glucose loads to prevent this from confounding the results. We demonstrate for the first time that kisspeptin modulates serum lipids and small molecules in humans. Several of these metabolites include classes of lipids (i.e. lysophosphatidylcholines, phosphocholines and sphingomyelins), which have been shown to be associated with insulin secretion.³⁶ This provides further evidence of kisspeptin's modulation of human metabolism, which (with further study) may provide important mechanistic data.

The principal limitation to the metabolite analysis approach used in the present study is that the measurements taken for each chemical species do not yield absolute quantities. Rather, they provide values that are proportional to the concentration of chemicals within the biological fluid, which are only useful in relative comparison within the experiment rather than in comparison to established concentration ranges. We have accounted for this limitation through the use of linear mixed effect models which rely solely on the differences observed in the relative abundance of individual metabolites to generate patterns of statistical significance. This allows the determination of significance and magnitude of metabolic changes.

In light of emerging evidence for neuroanatomical and functional connections between kisspeptin and key appetite-regulating neurones in the hypothalamus,^{15,16} and animal data which suggest a role for kisspeptin in energy homeostasis,²¹ we investigated the effect of kisspeptin on appetite in healthy men. Our data demonstrate kisspeptin had no effect on appetite and food intake in men. This is in keeping with rodent data showing that male *kiss1r* knockout mice have unaltered food intake.²¹

Our findings have pharmacological and potential therapeutic relevance as the plasma kisspeptin levels achieved in this study, which enhanced GSIS, are similar to those required to restore LH pulsatility in women with hypothalamic amenorrhea³ and trigger oocyte maturation in *in vitro* fertilisation protocols.² During IVGTTs, pharmacological kisspeptin administration increased insulin secretion and increased disposition index (IVGTT-DI). IVGTT-DI quantifies the ability of the β -cell to counter insulin resistance,³² with lower baseline IVGTT-DI values independently predicting conversion from normal glucose tolerance or impaired glucose tolerance to type 2 diabetes within 5 years.³² Therefore, our finding that kisspeptin increases GSIS and IVGTT-DI shows metabolically the beneficial effects of kisspeptin.

This is especially important as kisspeptin-based treatments are currently being developed to treat reproductive disorders²⁻⁵ and such treatments may therefore have additional potentially beneficial metabolic therapeutic applications. Our finding that kisspeptin administration also improves GSIS suggests that kisspeptin could have a dual therapeutic role in patients with diabetes to improve hypogonadism³³ as well as enhance insulin release, specifically only when hyperglycaemia occurs (thereby potentially avoiding the risks of hypoglycaemia associated with other diabetes treatments). Thus, our study lays the foundation for future studies exploring the effect of kisspeptin in the treatment of diabetes. Furthermore, both kisspeptin-54 and the smaller fragment, kisspeptin-10 (which may be produced by enzymatic breakdown) have been shown to enhance GSIS *in vivo* in humans in this study and *in vivo* in monkeys,¹¹ respectively. Therefore, metabolically active breakdown products of kisspeptin may serve to potentiate the metabolically beneficial effects of administered kisspeptin.

Our data demonstrating that pharmacological elevation of circulating kisspeptin levels results in a significant increase in GSIS in humans *in vivo* may also have potential physiological relevance. The circulating kisspeptin levels achieved by pharmacological administration of kisspeptin during this study are also observed in humans physiologically during pregnancy due to placental kisspeptin production (1–10 nM).⁴⁶ Therefore, the higher circulating kisspeptin levels during normal pregnancy may play a physiological role to enhance insulin secretion to protect the mother and foetus from increasing glucose levels and the development of gestational diabetes mellitus. In keeping with this hypothesis, pregnant women with diabetes have lower kisspeptin levels than pregnant women without diabetes.⁴⁷ In addition, previous rodent work has shown that selective ablation of the kisspeptin receptor, *kiss1r*, from pancreatic islet β -cells in pregnant mice results in impaired glucose tolerance and reduced insulin secretion.⁴⁸ Together these data suggest that elevated kisspeptin during pregnancy may play a positive physiological role in glucose homeostasis to protect against the development of gestational diabetes mellitus.

In summary, reproduction and metabolism are fundamental and interdependent aspects of mammalian physiology. This comprehensive study demonstrates that administration of the reproductive hormone kisspeptin to humans significantly increases GSIS *in vivo* and *in vitro* via actions on pancreatic islet cells, with associated alterations in serum metabolites associated with insulin secretion. We also show that kisspeptin does not affect appetite or food intake in healthy men.

This is the first human *in vivo* report of the effect of kisspeptin on β -cell function, metabolites and appetite, which is important for our understanding of the links between reproduction and metabolism in humans, as well as the ongoing development of kisspeptin-based treatments for common reproductive and potential metabolic disorders.

ACKNOWLEDGMENTS

This article presents independent research supported by the Imperial College Academic Health Sciences Centre, National Institute for Health Research Clinical Research Facility and Biomedical Research Centre (NIHR CRF and BRC) at Imperial College Healthcare NHS Trust. The Section of Endocrinology and Investigative Medicine is funded by grants from the Medical Research Council, Biotechnology and Biological

Sciences Research Council, NIHR, an Integrative Mammalian Biology Capacity Building Award, an FP7- HEALTH- 2009- 241592 EuroCHIP grant, and is supported by the NIHR BRC Funding Scheme. The National Phenome Centre is supported by the MRC and NIHR (grant number MC_PC_12025). Infrastructure support for this work was provided by the NIHR Imperial Biomedical Research Centre. The views expressed are those of the authors and not necessarily those of the MRC, the NHS, the NIHR or the Department of Health. C. I.-E. and R. R. are recipients of MRC Clinical Research Training Fellowships. A. N. C. is supported by the NHS and BRC. S. C. and P. C. E. are supported by funding from an NIHR Research Professorship. A. A. is supported by an NIHR Clinical Lecture-ship. L. Y. is a recipient of an Imperial College Healthcare Charity Fellowship. D. P. is supported by NIHR CLRN funding. T. T. is supported by grants from the MRC and Wellcome Trust. W. S. D. is supported by an NIHR Research Professorship (Grant number RP-2014-05-001). G. A. R. is supported by MRC Programme (MR/J0003042/1; MR/N00275X/1; MR/L020149/1 [DIVA]), Wellcome Trust Senior Investigator (WT098424AIA) and Diabetes UK Project (BDA11/0004210; BDA/15/0005275) grants.

The authors thank Drs Philippe Ravassard (Université Pierre et Marie Curie, Paris, France) and Raphael Scharfmann (INSERM U1016, Cochin Institute, Paris, France) for the provision of EndoC- β H1 cells, as well as David Berry and Ash Salam for their technical assistance in the identification of metabolites.

Conflict of interest

None declared.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Izzi-Engbeaya C, Comninou AN, Clarke SA, et al. The effects of kisspeptin on β -cell function, serum metabolites and appetite in humans. *Diabetes Obes Metab*. 2018;20:2800-2810. <https://doi.org/10.1111/dom.13460>