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GPR54 and Kisspeptins

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Abstract The G-protein coupled receptor GPR54 has an essential role in the initiation and maintenance of mammalian fertility. Humans and mice with mutations in GPR54 have hypogonadotropic hypogonadism characterized by absence of sexual maturation and low levels of gonadotropic hormones (LH and FSH). The ligand for GPR54 is encoded by the *KISS1* gene, which produces a 54-amino-acid peptide (metastin or kisspeptin-54) that can be cleaved into shorter peptides (kisspeptins 14, 13 and 10) with similar potencies. Kisspeptin administration stimulates gonadotropin release in several species by inducing GnRH secretion from hypothalamic GnRH neurons expressing GPR54. Kisspeptins are produced by neurons located in the AVPV and ARC regions of the hypothalamus. Expression of *Kiss1* in these neurons is differentially regulated by sex steroids providing a mechanism by which testosterone or estrogen can regulate GnRH release. The AVPV region is sexually dimorphic with highest expression of kisspeptin in females. Positive feedback by estrogen on expression of *Kiss1* in the AVPV region may be responsible for the pre-ovulatory LH surge during the estrus cycle. Central administration of kisspeptin to immature female rats can induce precocious activation of the gonadotropic axis, causing advanced vaginal opening, elevated uterus weight, increased serum levels of LH and estrogen and induce ovulation. Kisspeptins/GPR54 have also been implicated in regulating the estrus cycle of seasonal breeders and in the control of lactational amenorrhea. Expression of *Gpr54* and *Kiss1* have also been reported in several peripheral tissues including the pituitary, ovary, testes and the placenta raising the possibility that these genes may have additional functions in these tissues. Regulation of kisspeptin expression by peripheral factors such as leptin may be involved in coordinating metabolic status with the reproductive axis.

1 Introduction

Mammalian reproductive function is regulated by hormonal messengers and feedback loops within the hypothalamic-pituitary-gonadal axis (Fig. 1A). At puberty, neurons in the medial preoptic area of the hypothalamus initiate the pulsatile secretion of gonadotropin releasing hormone (GnRH) into the portal blood system for delivery to the anterior pituitary. Within the pituitary, GnRH stimulates gonadotropic cells to release the gonadotropic hormones luteinizing hormone (LH) and follicle stimulating hormone (FSH). The gonadotropins act on the gonads to stimulate synthesis of the sex steroids (testosterone and estrogen) which are required for spermato-

genesis and oogenesis. Hormonal feedback loops exist between the gonads, hypothalamus and the pituitary to regulate gonadotropin production (Fig. 1A).

Defining the physiological processes that initiate the pulsatile secretion of GnRH at puberty have proved elusive. The GnRH neurosecretory system is functional in neonatal primates with pulsatile gonadotropin secretions during the first months of life followed by suppression of hormone secretion until puberty. In primates, pre-pubescent GnRH secretion is suppressed by γ-aminobutyric acid (GABA) and removal of this suppression coincides with puberty. Loss of this suppression allows the reproductive axes of several species to respond to excitatory amino-acids, such as glutamate (Plant et al. 1989; Urbanski and Ojeda 1987). Conversely, antagonists of the glutaminergic NMDA receptor delay the onset of puberty in rats (Lopez et al. 1990; Urbanski and Ojeda 1990). New key molecules have now been identified

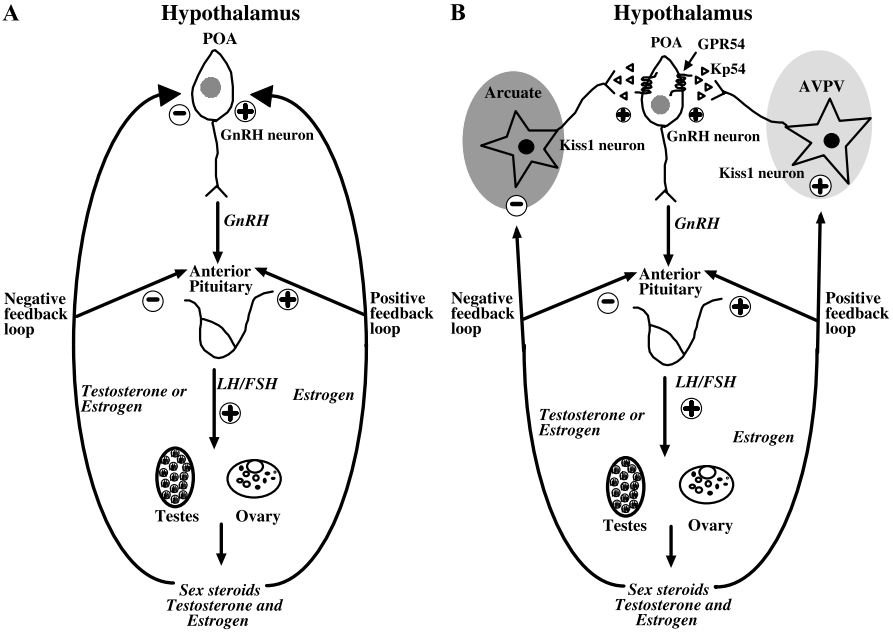

Fig. 1 Hormonal feedback loops in the Hypothalamic-Pituitary-Gonadal axis (**A**) and relationship to kisspeptin producing neurons (**B**). Kisspeptins act as a key mediator of the sex steroid feedback loops that regulate GnRH release from the hypothalamus. Kisspeptins act directly on GnRH neurons via the GPR54 receptor to stimulate GnRH release. Down regulation of kisspeptins in neurons of the arcuate nucleus provide the negative feedback action of sex steroids on GnRH release. Up regulation of kisspeptins by estrogen in neurons of the AVPV region in females stimulates GnRH release to provide the LH surge required for ovulation. GnRH, gonadotropin releasing hormone; LH, luteinizing hormone; FSH, follicle stimulating hormone, POA, preoptic area; AVPV, anteroventral periventricular nucleus

that are essential for awakening GnRH release at puberty. These molecules are the G-protein coupled receptor, GPR54, and peptide ligands for this receptor encoded by the *Kiss1* gene.

2 The GPR54 Receptor

GPR54 (also called *AXOR12* and *hOT7T175*) was originally amplified from a rat brain cDNA library using degenerate primers to conserved sequences within the third and seventh transmembrane domains of the G-protein coupled receptor family (Lee et al. 1999). *GPR54* consists of five exons and encodes a 398 (395 in mice and 396 in rats) amino-acid protein with slight homology (around 45% amino-acid identity in the transmembrane regions) to galanin receptors but with no functional interaction with galanin (Ohtaki et al. 2001). Chinese hamster ovary (CHO) cells transfected with a GPR54 expression plasmid produced a 75 kDa protein, which is larger than predicted suggesting possible post-translational modification by glycosylation at three potential sites in the extracellular amino-terminus or by carboxy-terminal palmitoylation (Lee et al. 1999; Muir et al. 2001).

GPR54 is most highly expressed in the human pituitary, pancreas and placenta with lower expression in peripheral blood leukocytes, smooth muscle of some blood vessels, testes, spleen, thymus, adrenal glands and lymph nodes (Funes et al. 2003; Mead et al. 2007; Muir et al. 2001; Ohtaki et al. 2001). In the adult brain, expression is found in the superior frontal gyrus, putamen, caudate nucleus, cingulate gyrus, nucleus accumbens, hippocampus, medulla pons and amygdala as well as the hypothalamus (Kotani et al. 2001; Lee et al. 1999; Muir et al. 2001).

3 Kiss1

A physiological ligand for the GPR54 receptor was identified by several groups in 2001 (Kotani et al. 2001; Muir et al. 2001; Ohtaki et al. 2001) and is encoded by the *KISS1* gene, which produces a 145 amino-acid protein that is proteolytic cleaved to generate the biologically active 54 amino-acid amidated protein, Kisspeptin 54 (Kp54) also known as metastin (Fig. 2). The carboxy-terminal region of Kp54 is responsible for receptor binding and this region is the most conserved between species (Fig. 3). Carboxy-terminal peptides of 14, 13 or 10 amino-acids (Kp14, Kp13 and Kp10) show similar activities in vitro to Kp54 (Kotani et al. 2001; Muir et al. 2001; Ohtaki et al. 2001). Binding of kisspeptin to GPR54 stimulates the G-protein Gq to activate phospholipase C and increases intracellular IP_3 and Ca^{2+} and activate the ERK and

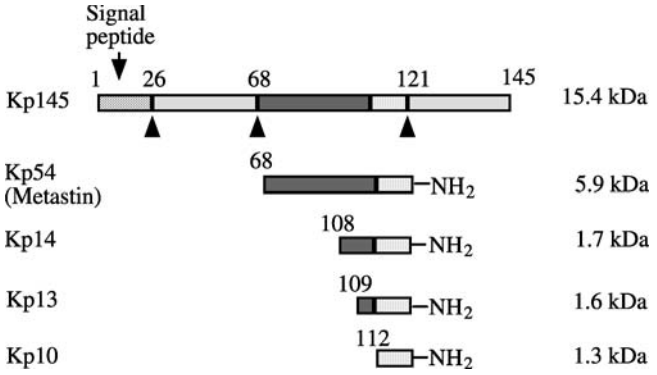

Fig. 2 Processing of KISS1 to produce kisspeptins. The primary protein product of the *Kiss1* gene is cleaved (*arrowheads*) to produce smaller amidated peptides (kisspeptins, Kp) capable of binding to GPR54. All biologically active peptides contain a common carboxy terminal decapeptide sequence (*stippled*)

Human	D	L	P	N	Y	N	W	N	S	F	G	L	R	F-NH_2
Chimp	D	L	P	N	Y	N	W	N	S	F	G	L	R	F-NH_2
Mouse	D	L	***S***	***T***	Y	N	W	N	S	F	G	L	R	***Y***-NH_2
Rat	D	***M***	***S***	***A***	Y	N	W	N	S	F	G	L	R	***Y***-NH_2
Sheep	D	***V***	***S***	***A***	Y	N	W	N	S	F	G	L	R	***Y***-NH_2

Fig. 3 Species comparison of kisspeptin sequences. Amino-acids are indicated by the single letter code and divergence from the human sequence indicated in *bold italic letters*

p38 MAP kinase pathways. Carboxy-terminal amidation is required for all the peptides to stimulate intracellular Ca^{2+} increase (Muir et al. 2001; Ohtaki et al. 2001).

Kiss1 is highly expressed by syncytiotrophoblast cells in the placenta (Horikoshi et al. 2003). Consequently, plasma kisspeptin levels are raised in patients with molar pregnancies which contain trophoblastic tissue (gestational trophoblastic neoplasia) (Dhillo et al. 2006). Low levels of Kisspeptin-54 are present in the plasma of males and females but this rises dramatically during pregnancy the physiological significance of which is not known (Horikoshi et al. 2003). Lower levels of *Kiss1* are expressed in the testes, liver, pancreas and small intestine (Ohtaki et al. 2001).

4 Role in Metastasis

Kiss1 was originally identified as a differentially expressed cDNA that suppressed the metastatic spread of the human melanoma cell line C8161 after transfection of human chromosome 6 (Lee et al. 1996). As the human *Kiss1* gene is located on chromosome 1, this suggests that a positive regulator of *Kiss1* is located on chromosome 6. Transfection of a chromosome 6 deletion into C8161 cells indicated that this regulator maps between 6q16.3-q23 (Miele et al. 2000). The Sp1-coactivator protein DRIP-130 is a candidate for this regulator as it maps within this region and can increase *Kiss1* transcription in cancer cell lines when co-expressed with the transcription factor Sp1 (Mitchell et al. 2007). Subsequently *Kiss1* has been shown to suppress metastasis of additional human cancer cell lines in xenograft animal models including breast (Lee and Welch 1997) and ovarian carcinomas (Jiang et al. 2005). Reduced expression of *Kiss1* also correlates with increased metastatic potential and poorer prognosis of several human cancers including melanomas (Shirasaki et al. 2001), bladder carcinomas (Sanchez-Carbayo et al. 2003), hepatocarcinomas (Ikeguchi et al. 2003), gastric cancers (Dhar et al. 2004), pancreatic cancer (Masui et al. 2004), oesophageal squamous cell carcinomas (Ikeguchi et al. 2004), malignant pheochromocytomas (Ohta et al. 2005), and breast cancers (Stark et al. 2005). Conversely, some thyroid cancers that do not show loss of *Kiss1* expression are associated with increased expression of the GPR54 receptor (Ringel et al. 2002). Thus, changes in expression of either *Kiss1* or *GPR54* may influence the severity and progression of some types of cancer.

The mechanism by which kisspeptins suppress metastasis is not clear but may not require a change in cell proliferation. The effect of kisspeptins on cell proliferation is unresolved, with some groups reporting no effect (Bilban et al. 2004; Lee et al. 1996) while others report inhibition of cell proliferation (Kotani et al. 2001; Stafford et al. 2002). Kisspeptins also inhibit the migration in culture of cell lines expressing GPR54 such as transfected CHO cells (Ohtaki et al. 2001; Stafford et al. 2002) or primary human trophoblast cells (Bilban et al. 2004). Migratory inhibition may be caused by increased formation of focal adhesion points (Ohtaki et al. 2001) or reduced secretion of proteases such as MMP-2 (Bilban et al. 2004). The inhibitory effect of kisspeptin on migration of the human ovarian cancer cell line SKOV3 can be overcome by phorbol ester stimulation of protein kinase Cα (Jiang et al. 2005) suggesting that continuous GPR54 signalling could reduce cell migration by inhibiting PKCα activity. Kisspeptins also inhibit the intracellular signalling cascade of the pro-metastatic G-protein coupled receptor CXCR4 (Navenot et al. 2005). Metastasis suppression requires kisspeptin secretion as melanoma lines expressing non-secreted forms of the protein are not inhibited (Nash et al. 2007) suggesting an autocrine or paracrine mechanism of action.

5
Role in Reproduction

5.1
GPR54 Mutations in Hypogonadotropic Hypogonadism

5.1.1
Humans

The crucial role that GPR54 plays in human fertility was originally identified by two independent research groups that found loss of function mutations in patients with idiopathic hypogonadotropic hypogonadism (IHH) (de Roux et al. 2003; Seminara et al. 2003). IHH is a clinical condition characterized by absence of pubertal sexual development and low sex steroid and gonadotropin levels in the blood. Some individuals with IHH also have an inability to smell (anosmia, Kallmann syndrome) due to a failure of olfactory bulb neuron development and migration (including GnRH neurons) but individuals with GPR54 mutations are all normosmic. Mutations in several genes can cause hypogonadotropic hypogonadism including the GnRH receptor, FGFR1, DAX-1 and KAL1 although not all cases of IHH have had a specific genetic defect ascribed to them (Iovane et al. 2004).

Most *GPR54* mutations have been identified in individuals from consanguineous marriages between first cousins. De Roux and colleagues studied a family of eight children of whom five were affected by IHH (de Roux et al.

Table 1 GPR54 mutations associated with hypogonadotropic hypogonadism

Mutation	Location	Amount of normal cell signalling	Refs.
Δ155 (Intron 4–142Ex5)	3rd Extracellular Loop termination	Not Described	(de Roux et al. 2003)
L146S (443T > C)	2nd Internal Loop	35%	(Seminara et al. 2003)
R331X (991C > T)	Proximal part COOH terminus	33% 60%	
X339R (1195T > A)	COOH terminal extension		
C223R (667T > C)	5th TMH	20%	(Semple et al. 2005)
R297L (891G > T)	3rd External Loop	85%	
1001–1002insC	Proximal part COOH terminus	Not Described	(Lanfranco et al. 2005)
L102P	1st Extracellular Loop	10%	(de Roux et al. 2003) (Tenenbaum-Rakover et al. 2007)

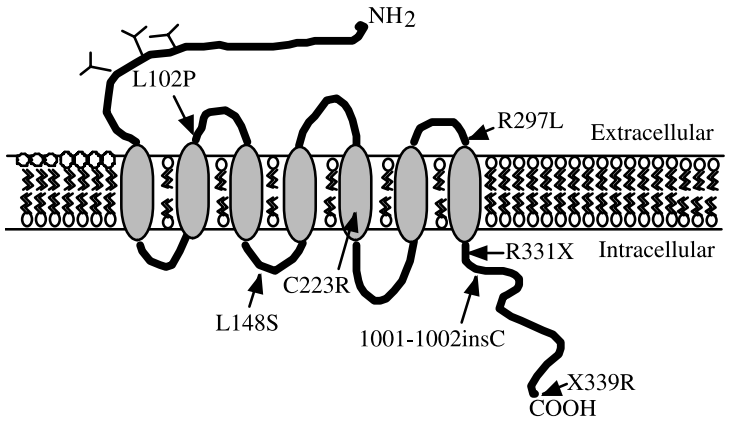

Fig. 4 Location of GPR54 mutations. GPR54 is a G-protein coupled receptor with seven transmembrane domains

2003). A genome-wide linkage scan localized the mutation to a small region of chromosome 19 and candidate genes in this region were sequenced for mutations. A 155 bp deletion was found starting in intron 4 and extending 142 bases into exon 5 of the *GPR54* gene that would produce a truncated protein incapable of G-protein coupling. A missense mutation (leucine to proline at position 102) was also identified in a sporadic case of IHH. Seminara and colleagues used a similar approach to identify GPR54 mutations in a family from Saudi Arabia (Seminara et al. 2003) and found a nonsense mutation leading to a premature stop codon and a nonstop mutation (X399R) that would extend the length of the GPR54 protein. Subsequently, additional mutations in *GPR54* have been identified (Table 1). These mutations are found in different locations of the GPR54 protein and might reduce signalling by different mechanisms (Fig. 4). Mutations within extracellular loops of GPR54 (L102P and R297L) might alter interaction with kisspeptins, while those at the C-terminus (and truncations) would remove interaction with the Gq protein. Indeed, most GPR54 mutations have been expressed in heterologous cell systems and show a reduction in cell signalling compared to wild-type (Table 1).

5.1.2 Mice

The role that GPR54 plays in regulating the fertility of other mammals has been confirmed by the independent generation of three different transgenic mouse lines with disruptions of the *Gpr54* gene (Funes et al. 2003; Kauffman et al. 2007b; Lapatto et al. 2007; Messager et al. 2005; Seminara et al. 2003). In the mice generated by Colledge and colleagues, a segment spanning intron 1 and parts of exon1 and exon 2 of the *Gpr54* coding sequence have been removed and replaced with an IRES-LacZ sequence that allows

the expression pattern of the *Gpr54* gene to be visualized by detection of β-galactosidase activity. Mutant animals of both sexes fail to undergo pubertal sexual development and are sterile. Mutant males have small testes, severe disruption of spermatogenesis and fail to develop secondary sex glands such as the seminiferous vesicles and preputial glands. Mutant females have no estrus cycle, thread-like uteri and ovaries with no mature antral follicle formation. The mammary glands do not show the normal pubertal development of a branched epithelial duct system. Both sexes have low sex steroids and gonadotropic hormones but retain functional pituitary responses to GnRH. Importantly, the mutant mice have normal hypothalamic GnRH content and correct localization of GnRH neurons suggesting a primary defect in GnRH release rather than a developmental defect in GnRH migration.

Mice with a disrupted *Kiss1* gene have also been generated and these show similar reproductive defects to the *Gpr54* mutants (d'Anglemont de Tassigny et al. 2007; Lapatto et al. 2007). *Kiss1* mutant mice have abnormal pubertal maturation of the reproductive system, hypogonadotropic hypogonadism and low sex steroid levels but retain the ability to secrete gonadotropic hormones after kisspeptin injection. It has been reported that the *Kiss1* mutant mice generated by Lapatto and colleagues (*Kiss1*^{tm1MGH}) have a more variable phenotype than *Gpr54* mutant mice (Lapatto et al. 2007). Around half of the *Kiss1*^{tm1MGH} mice show vaginal opening and ovary weights similar to wild-type. In contrast, the *Kiss1* mutant mice generated by d'Anglemont de Tassigny and colleagues (*Kiss1*^{tm1PTL}) do not show vaginal opening even up to 6 months of age. The reason for this difference is unknown but may reflect the slightly different genetic background of the two lines with the *Kiss1*^{tm1MGH} mice on a 129S1/SvIMJ background and the *Kiss1*^{tm1PTL} mice on a 129S6/SvEv background. These mutant mice show that GPR54 and kisspeptins are both essential for the activation of the hypothalamic-pituitary-gonadal axis at puberty.

5.2 Hypothalamic Expression Pattern

The expression pattern of *Gpr54* and *Kiss1* in the hypothalamus is consistent with the function of these genes in the central control of reproduction. The hypothalamus is located just above the brain stem and regulates many important physiological processes including body temperature, hunger, thirst, circadian cycles and reproduction. The hypothalamus is arranged either side of the third ventricle and divided into three functionally distinct zones (periventricular, medial, and lateral). The periventricular and medial zones contain most of the hypothalamic neuronal cell bodies with the lateral zone containing fewer neurons. *Kiss1* and *Gpr54* are expressed in discrete neuronal populations (nuclei) in the hypothalamus (Fig. 5). *Kiss1* expression in rodents has been mapped by in situ

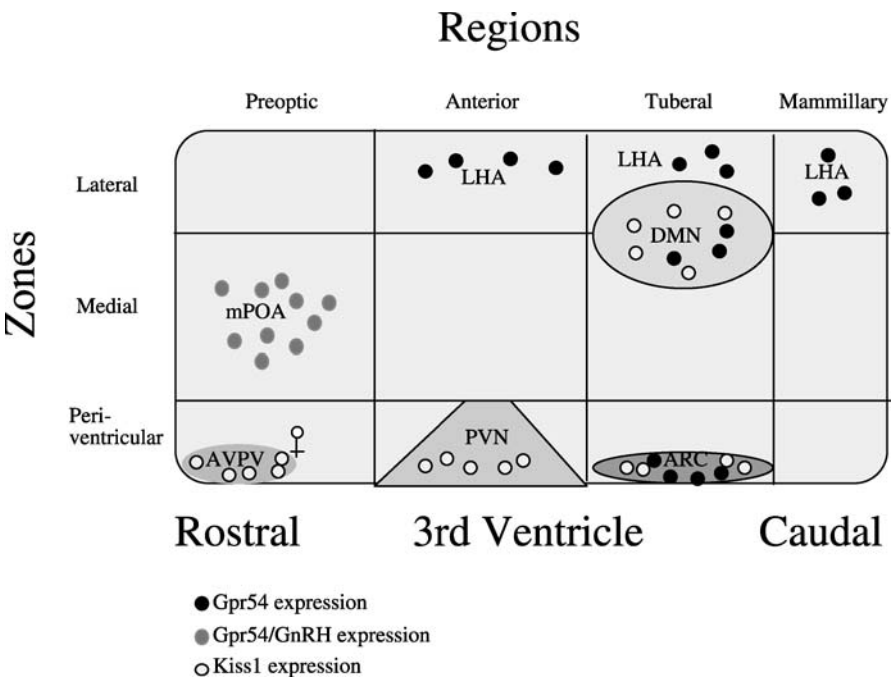

Fig. 5 Consensus expression of *Gpr54* and *Kiss1* in cell bodies in the rodent hypothalamus. Schematic representation of the morphological organization of the hypothalamus. Expression pattern based on in situ hybridization data and KISS1 protein immunohistochemistry. The AVPV region is sexually dimorphic with higher *Kiss1* expression in the female. LHA, lateral hypothalamic area; mPOA, medial preoptic area; DMN, dorsomedial nucleus; AVPV, anteroventral periventricular nucleus; PVN, periventricular nucleus; ARC, arcuate nucleus

hybridization to the arcuate nucleus (ARC), anteroventral periventricular nucleus (AVPV) and the periventricular nucleus (PVN) (Gottsch et al. 2004; Irwig et al. 2004). Immunohistochemical detection of KISS1 protein has not always given concordant results with the in situ expression data possibly due to species differences and antibody specificity. Several anti-KISS1-antibodies have been used by different groups including a commercially available one raised against the human kisspeptin-10 sequence [YNWNSFGLPF-NH_2 (Brailoiu et al. 2005; Pompolo et al. 2006)], a rabbit polyclonal against mouse kisspeptin-10 [YNWNSFGLRY-NH_2 (Franceschini et al. 2006; Clarkson and Herbison 2006)] and a mouse monoclonal against a slightly longer rat kisspeptin sequence [EKDMSAYNWNSFGLRY-NH_2 (Kinoshita et al. 2005)]. The consensus is that kisspeptin immunoreactive cell bodies are found in the ARC, AVPV and PVN of most species (Brailoiu et al. 2005; Clarkson and Herbison 2006; Franceschini et al. 2006; Kinoshita et al. 2005; Pompolo et al. 2006). Kisspeptin-10 immunoreactive cell bodies have

also been found in the dorsomedial hypothalamus of the rat (Brailoiu et al. 2005), mouse (Clarkson and Herbison 2006) and sheep (Franceschini et al. 2006) using two different antibodies but expression in this region has not been found by in situ hybridization. Kisspeptin immunoreactive fibres have been shown to innervate the preoptic area (POA) in sheep (Franceschini et al. 2006; Pompolo et al. 2006) and make intimate connection with GnRH neurons in rodents (Kinoshita et al. 2005; Clarkson and Herbison 2006). Transgenic mice with a *LacZ* targeted *Kiss1* locus have allowed us to visualize *Kiss1* expression by β-galactosidase staining (d'Anglemont de Tassigny et al. 2007). These mice have β-galactosidase expression in the expected regions of the ARC, AVPV and PVN but also show staining in the medial mamillary nucleus which has not previously been reported to express *Kiss1* (d'Anglemont de Tassigny et al. 2007; X. d'AdeT, unpublished).

Gpr54 was first shown to be expressed in the hypothalamus by qRT-PCR at levels approximately 500x less than the housekeeping gene *Gapdh* (Muir et al. 2001). The cellular distribution of *Gpr54* expression in the hypothalamus of rodents as defined by in situ hybridization is localized to the diagonal band of Broca, the medial septum, preoptic areas and the anterior and lateral hypothalamus (Han et al. 2005; Irwig et al. 2004). Of particular significance is the finding that the majority of GnRH neurons in mammals (up to 90%) also express *Gpr54* (Han et al. 2005; Irwig et al. 2004; Messager et al. 2005). Gpr54 expression has also been found in GnRH neurons prior to birth with expression at the 18th day of gestation in rat fetuses (Quaynor et al. 2007). Gpr54 expression by GnRH neurons extends to the cichlid fish suggesting a conservation of function beyond mammals and into other vertebrates (Parhar et al. 2004).

5.3 Stimulation of Gonadotropin Secretion

The function of kisspeptins in vivo has been studied by injection of these peptides into several animal species. These studies have shown that kisspeptins play a crucial role in stimulating gonadotropin release in rats (Castellano et al. 2006b; Irwig et al. 2004; Matsui et al. 2004; Navarro et al. 2004a, 2005a,b; Thompson et al. 2004), mice (Gottsch et al. 2004; Messager et al. 2005), sheep (Messager et al. 2005), primates (Plant et al. 2006; Shahab et al. 2005) and humans (Dhillo et al. 2005) after systemic (intravenous, intraperitoneal or subcutaneous) or intracerebroventricular (ICV) delivery. Kisspeptins are extremely potent agonists of gonadotropin secretion with as little as 1 fmol producing significant release of LH after ICV injection in mice (Gottsch et al. 2004). This is considerably more effective than other substances that stimulate GnRH release (eg., glutamate) and the kisspeptin responses are particularly long lasting often maintaining LH release over several hours. Kisspeptins activate GnRH neurons indicated by an increase in *c-fos* immunoreactivity (Irwig et al. 2004; Matsui et al. 2004) probably by a direct action as GnRH neu-

rons express the GPR54 receptor (Han et al. 2005; Irwig et al. 2004; Messager et al. 2005). Significantly, responses to kisspeptins are absent in *Gpr54* mutant mice showing that GPR54 directly mediates GnRH secretion (Messager et al. 2005). In addition, we have directly shown GnRH secretion in response to Kisspeptin injection in sheep (Messager et al. 2005). Thus, the principle function of kisspeptins in vivo are to act via the GPR54 receptor to stimulate GnRH release and activate the pituitary gonadal axis.

Kisspeptin stimulation of the GPR54 receptor in GnRH neurons may be subject to negative feedback modulation by GnRH. GPR54 and the GnRH-receptor have been shown to form a close association by bioluminescence resonance energy transfer in HEK-293 cells (Quaynor et al. 2007). Moreover, kisspeptin-10 mediated enhancement of GnRH release from the GnRH neuronal cell line GT1-7 can be inhibited by GnRH itself. This suggests that the long recognized ability of GnRH to suppress its own release may be mediated by the GnRH-receptor altering kisspeptin/GPR54 signalling.

As well as these acute effects of kisspeptins on gonadotropin release, the effects of continuous delivery have also been studied. The first chronic delivery study was performed using castrated juvenile male rhesus monkeys intravenously infused with 100 μg/h human Kisspeptin-10 for 4 days (Seminara et al. 2006). Under these conditions, LH secretion was initially stimulated over a 3h period followed by suppression. The monkeys retained LH responses to NMDA and GnRH during the infusion period demonstrating the functional integrity of GnRH neurons and pituitary gonadotrophs. Thus, continuous kisspeptin infusion desensitizes the GPR54 receptor rather than other components of the hypothalamic-pituitary axis in juvenile monkeys. These studies have been extended using non-castrated adult male monkeys (Ramaswamy et al. 2007). A similar increase in LH release immediately after Kisspeptin-10 infusion was found, followed by a decrease at longer time points. In contrast to the juvenile monkeys, the LH responses to NMDA and GnRH injection were reduced, suggesting that in adult monkeys continuous kisspeptin administration desensitizes not only GPR54 but also the GnRH receptor in the pituitary. In rats, chronic subcutaneous administration of Kisspeptin-54 initially increased LH and testosterone after 1 day but this effect was lost by day 2 (Thompson et al. 2006). Longer-term administration of Kisspeptin-54 for 13 days decreased testicular weight and led to degeneration of seminiferous tubules (Thompson et al. 2006).

An early report also claimed that intravenous injection of Kisspeptin-10 increased plasma oxytocin levels in female rats (Kotani et al. 2001). The significance of this observation is not clear and no subsequent reports have confirmed this data. Oxytocin is synthesized by magnocellular neurons in the supraoptic and paraventricular nuclei of the hypothalamus and transported axonally to the posterior pituitary. Oxytocin has a principle role in parturition, lactation and maternal behavior none of which have been yet determined in *Kiss1* mutant mice because of their infertility.

5.4 Activation at Puberty

The absence of puberty in humans and mice with mutations in GPR54 shows that this protein is required for pubertal development but does not prove that GPR54 is a key regulator of this event. Several lines of evidence however, indicate that GPR54 and kisspeptins are more than just downstream mediators of puberty but rather that they are key molecules involved in switching on the HPG axis. *Kiss1* and *Gpr54* expression increase coincidentally with puberty in several species. In the rat, *Kiss1* and *Gpr54* both increased in the hypothalamus as a whole at puberty (Navarro et al. 2004a). More detailed, in situ hybridization studies showed that this increase in *Kiss1* was confined to the AVPV region with little change in the ARC of male mice (Han et al. 2005). Consistent with this, the number of kisspeptin immunoreactive neurons increased in the AVPV/PVN of the mouse during puberty but little change was found in the ARC (Clarkson and Herbison 2006). In primates, *KISS1* and *GPR54* mRNA levels increased in the hypothalamus during the transition from juvenile to mid-puberty stages in intact females (Shahab et al. 2005). In castrated males, *KISS1* expression was 3-fold greater in the pubertal group than in the juvenile animals while *GPR54* expression did not change. Thus, an increase in *Kiss1* expression around the time of puberty is a consistent observation in many species. Consonant with this pubertal increase in *Kiss1* expression, exogenous injection of kisspeptins can advance pubertal development. Chronic administration of Kisspeptin-10 to immature female rats advanced vaginal opening (a sign of puberty) by 5 days (Navarro et al. 2004b) and repetitive injection of Kisspeptin-10 in juvenile primates induced a precocious chain of GnRH pulses similar to those occurring at puberty (Plant et al. 2006).

As well as the expression changes that occur to *Kiss1* and *Gpr54*, it is likely that other functional and anatomical changes are required for proper activation of GnRH release at puberty. For example, the sensitivity of GnRH neurons to kisspeptins alters during pubertal development. Kisspeptins caused a rapid depolarization of over 90% of GnRH neurons in adult mice but only around 30% of neurons in juvenile animals, even though the expression of *Gpr54* was very similar between the age groups (Han et al. 2005). This difference in the sensitivity of GnRH neurons was confirmed in vivo using low doses of kisspeptin which elicited LH release in adults but not juvenile mice after ICV delivery (Han et al. 2005). Similar changes in sensitivity have been found in male rats where ICV injection of low doses of Kisspeptin-10 (1 or 10 pmol) produced better LH secretion in 40-day-old animals than juvenile 15-day-old animals (Castellano et al. 2006b). This developmental change in sensitivity of GnRH neurons may also explain why the immortalized GnRH cell line, GT1-7 does not respond to kisspeptins even though it expresses *Gpr54* (Nazian 2006). It is possible that the GT1-7 line

represents an immature GnRH cell type which may be useful in studying the mechanism by which GnRH cells become responsive to kisspeptins. It should also be remembered however, that there are also changes that occur to the neuronal circuitry at puberty that may be required for kisspeptin signalling. For example, connections between kisspeptin immunoreactive fibres and GnRH neurons increase across post-natal development in male and female mice (Clarkson and Herbison 2006).

5.5 Kiss1 Regulation by Sex Steroids

It is well established that GnRH and gonadotropin secretion are regulated by sex steroids but the mechanism by which this is achieved remains largely unknown. Direct action of estrogen on GnRH neurons is unlikely as they do not express the estrogen receptor alpha (ERα). It now seems likely that *Kiss1* expressing neurons integrate the feedback signals from gonadal steroids to GnRH neurons. *Kiss1* neurons make direct contact with GnRH neurons (Kinoshita et al. 2005; Clarkson and Herbison 2006) and *Kiss1* expression is regulated by sex steroids in a manner consistent with feedback control (Fig. 1B). Gonadectomy of male and female rats increases hypothalamic *Kiss1* expression as measured by RT-PCR, while steroid replacement abolishes this increase (Navarro et al. 2004a). This sex steroid regulation extends to higher primates as testosterone treatment of castrated adult male rhesus monkeys reduces *Kiss1* expression in the mediobasal hypothalamus (Shibata et al. 2007) and ovariectomy of young cynomolgus monkeys increases expression of *Kiss1* in the infundibular nucleus which can be prevented by estrogen replacement (Rometo et al. 2007). In post-menopausal women of average age 72 years with negligible estrogen production, *Kiss1* expression was significantly higher than in pre-menopausal women of average age 32 years (Rometo et al. 2007). This increase in kisspeptin expression may account for the elevation in gonadotropin secretion that occurs after menopause.

Subsequent studies by in situ hybridization and immunohistochemistry have provided more details about the effects of sex steroids on *Kiss1* expression in specific hypothalamic nuclei. In mice, *Kiss1* expression is decreased in the AVPV and PVN and increased in the ARC after gonadectomy (Smith et al. 2005a,b). These changes are eliminated by testosterone (in males) or estrogen (in females) replacement. In sheep, ovariectomy increases expression of *Kiss1* mRNA mainly in the ARC and to a lesser extent the POA (Smith et al. 2007) but immunoreactive kisspeptin only significantly increases in the ARC (Pompolo et al. 2006). *Kiss1* expression in the ARC was returned to the level found in intact sheep by estrogen treatment but progesterone also partially returned the level to normal (Smith et al. 2007). Whether progesterone has an effect on *Kiss1* expression in rodents has not yet been determined.

The effects of gonadal steroids on the expression of *Kiss1* in the hypothalamus would be expected to be mediated by steroid hormone receptors. Indeed, in male mice, around 65% of *Kiss1* neurons in the ARC also express the androgen receptor (AR) and around 90% express the estrogen receptor alpha (ERα) (Smith et al. 2005b). Co-expression data in the AVPV and PVN regions were not given for male mice. In female mice, the majority of *Kiss1* neurons in the ARC, AVPV, PVN express ERα and between 25–40% express ERβ (Smith et al. 2005a). In rats, around 60–70% of *Kiss1* neurons in the ARC and the AVPV express ERα with less (10–20%) expressing ERβ (Smith et al. 2007). In sheep, around 90% of *Kiss1* neurons in the ARC express ERα and 50% in the POA (Franceschini et al. 2006). 86% of *Kiss1* neurons in the sheep ARC also express the progesterone receptor (Smith et al. 2007). Whether *Kiss1* neurons express the progesterone receptor in rodents had not been reported. *Kiss1* neurons in the ARC nucleus of the ewe also express the neuropeptides dynorphin A and neurokinin B (Goodman et al. 2007). Dynorphin A is a 17 amino-acid opioid-like peptide and neurokinin B is a 10 amino-acid peptide of the tachykinin family. Both peptides have been implicated in regulating GnRH release so their co-expression in *Kiss1* neurons may have an important role in modulating the kisspeptin control of GnRH release.

The role of each type of steroid receptor in mediating sex steroid regulation of *Kiss1* expression was assessed by analysis of transgenic mice with mutations in these receptors. Male mice with mutations in either the ERα or the AR still show testosterone-mediated changes in *Kiss1* expression in the ARC, suggesting that this regulation is mediated by both receptor types (Smith et al. 2005b). This observation is also supported by the fact that the effects of testosterone on *Kiss1* expression in castrated mice are completely mimicked by estrogen treatment but only partially mimicked by dihydrotestosterone which cannot be aromatized to estrogen (Smith et al. 2005b). Thus, testosterone modulates *Kiss1* expression in the ARC through the AR and also the ERα after aromatization to estrogen. Female mice with a defective ERα no longer show regulation of *Kiss1* in the ARC and the AVPV, while ERβ mutant mice continue to show estrogen regulation (Smith et al. 2005a). Thus, in female mice ERα but not ERβ, has a crucial role in mediating the estrogen regulation of *Kiss1* expression in the hypothalamus.

The way in which *Kiss1* transcription is regulated is starting to be unravelled. The transcription factors AP-2α and Sp1 act synergistically to positively regulated *Kiss1* expression in breast cancer cell lines (Mitchell et al. 2006). The activity of Sp1 also requires expression of the co-activator protein DRIP-130 (Mitchell et al. 2007). The minimal promoter region that confers estrogen responsiveness on human *Kiss1* expression has been mapped (Li et al. 2007). While the highest induction of promoter activity to estrogen was found for a 1000 bp fragment immediately upstream of the transcription start site, a significant response to estrogen was also obtained with the most proximal

190 bp sequence. This 190 bp region does not actually contain consensus estrogen response elements for direct ERα binding but has four Sp1 binding sites. Sp1 and ERα form a complex to mediate the estrogen-induced activation of the *Kiss1* promoter. All four Sp1 binding sites contribute to the basal promoter activity while the two Sp1 binding sites closest to the transcriptional start site function together to allow estrogen stimulation (Li et al. 2007).

5.6 Kiss1 Expression During the Estrus Cycle

As expected from a gene regulated by sex steroids, *Kiss1* expression varies during the estrus cycle of several species and also shows fluctuations with the breeding season. Given the potency with which kisspeptins stimulate GnRH secretion, it is generally thought that these changes in expression are driving these reproductive cycles rather than simply following them. Significantly, *Kiss1* expression in the AVPV and the ARC regions of the rat hypothalamus show opposite changes during the estrus cycle, probably reflecting the different contributions of these regions in the control of GnRH release during the cycle. *Kiss1* expression is at its peak in the AVPV region during the evening of proestrus (the stage leading up to ovulation) in rats while expression in the ARC is declining at this point (Smith et al. 2006b). *Kiss1* neurons are specifically activated in the AVPV region at proestrus as indicated by an induction in *c-fos* expression (Smith et al. 2006b). This increase in *Kiss1* expression in the AVPV region coincides with the pre-ovulatory LH surge and is also increased during a steroid-induced LH surge in ovariectomized rats (Smith et al. 2006b). Inhibition of kisspeptin action in the POA by local injection of monoclonal antibody abolished the pro-estrous LH surge and inhibited estrous cyclicity in rats (Adachi et al. 2007; Kinoshita et al. 2005).

Consistent with the AVPV nucleus mainly operating in female rodents to induce the LH surge, *Kiss1* expression in this region is sexually dimorphic with female rats having more *Kiss1* neurons than male rats (Kauffman et al. 2007a). This difference is established perinatally by an androgen-mediated reduction in *Kiss1* expression since neonatally androgenized females show male patterns of *Kiss1* expression in the AVPV region at adulthood. The AVPV region of the rodent has been recognized as sexually dimorphic for several years with differences in the number of dopaminergic neurons (Simerly 1998) but the *Kiss1* neurons in this region are distinct from these dopaminergic neurons (Kauffman et al. 2007a). The androgenization process that establishes these sexual dimorphisms requires GPR54, since *Gpr54* knock-out male mice have dopaminergic and *Kiss1* neurons in the AVPV region similar in number to those found in females (Kauffman et al. 2007b). *Gpr54* mutant mice also lack an olfactory-mediated preference for female mice. Thus, GPR54/kisspeptin signalling probably acts during perinatal development to regulate the GnRH-

mediated androgen secretion necessary for the proper development of several sexually dimorphic traits.

Similar changes in *Kiss1* expression occur during the estrus cycle of the sheep but in this species the region of the hypothalamus that controls the pre-ovulatory LH surge is the ARC nucleus. Accordingly, *Kiss1* expression increases in the caudal region of the ARC nucleus in ewes to be highest at the late-follicular stage just before ovulation (Estrada et al. 2006).

5.7 Seasonal Breeding

Kisspeptins probably also regulate the seasonal breeding cycle of species that show circannual changes in reproductive capacity. Many species show seasonal breeding patterns to ensure that offspring are born at a time when environmental conditions are optimal for survival. For example, sheep usually breed in the autumn to give birth in the spring. During the non-breeding season, GnRH secretion is reduced and the estrous cycle shut down. An examination of *Kiss1* expression in the ARC nucleus of ovariectomized ewes by in situ hybridization has found that expression is highest during the breeding season and decreases around 50% in the non-breeding season (Smith et al. 2007). Importantly, because these changes occurred in ovariectomized sheep, this change in *Kiss1* expression is steroid-independent providing further support that kisspeptins drive these changes in reproductive function rather than follow them. Interestingly, it is the number of *Kiss1* positive cells that changes rather than the expression level per cell suggesting that around 50% of the *Kiss1* cells in the ARC do not change expression with breeding season. Why these remaining cells are not capable of driving GnRH release is not known but perhaps they do not make appropriate connections to GnRH neurons.

Siberian hamsters also show seasonal breeding and have been used to examine the effects of photoperiod on reproduction. These hamsters breed during long-day periods and become anestrus during short-days. Kisspeptin immunoreactive neurons were significantly reduced in the AVPV of non-breeding short-day hamsters compared to those kept under long days (Greives et al. 2007). In contrast, kisspeptin immunoreactive neurons dramatically increased in the ARC of short-day animals. These changes were not found in a polymorphic hamster line that does not show seasonal breeding. All hamsters responded to kisspeptin injection irrespective of reproductive state suggesting that the photoperiodic regulation of GnRH release is regulated by local kisspeptin production in the hypothalamus. The regulatory mechanisms that control these seasonal changes in *Kiss1* expression have not been identified. Some changes in *Kiss1* expression may reflect seasonal fluctuations in sex steroid levels but it is likely that other molecules that respond to photoperiodism, such as melatonin, may also play a role.

5.8 Lactation

Suppression of ovulation by lactational suckling is found in most mammalian species due to inhibition of GnRH/LH secretion although the precise mechanism is unknown (McNeilly 2001). Kisspeptins have now been implicated in lactational amenorrhea. Roa et al. (2006) reported a differential sensitivity to low doses of Kisspeptin-10 with lactating rats requiring higher doses of kisspeptins to secrete LH than rats in diestrus (Roa et al. 2006). This observation has been extended by Yamada et al. (2007) who studied the expression level of *Kiss1* in micro-dissected regions of the hypothalamus during lactation by qRT-PCR and immunohistochemistry. A significant reduction in *Kiss1* mRNA and protein expression was found in the ARC-ME region in lactating rats that was independent of sex steroids (Yamada et al. 2007). *Kiss1* expression in the AVPV was very low in both lactating and non-lactating rats with no significant difference between the groups. ICV injection of Kisspeptin-54 stimulated LH secretion indicating that the signalling pathways downstream of kisspeptins are functional in lactating rats. These data are consistent with modulation of *Kiss1* expression in the ARC controlling the suppression of ovulation during lactational suckling.

6 Kisspeptin Action at Non-Hypothalamic Sites

6.1 Pituitary

The role that kisspeptin/Gpr54 signalling plays in regulating hormonal release from the pituitary is equivocal. The pituitary has one of the highest levels of *GPR54* expression of all human tissues (Kotani et al. 2001; Muir et al. 2001) although the specific cell types expressing GPR54 have not been determined. Whether kisspeptins can act on the pituitary to stimulate hormone release is contentious. Navarro and colleagues have reported that mouse Kisspeptin-10 can stimulate LH secretion from adult male rat pituitary explants in a dose-dependent manner although this effect was 4-fold less potent than with GnRH (Navarro et al. 2005b). Similar effects have been found using dispersed pituitary cells from peripubertal male and female rats (Gutierrez-Pascual et al. 2007). In these studies, Kisspeptin-10 stimulated a rise in cytoplasmic Ca^{2+} levels in 63% of gonadotrophs. Weak Kisspeptin-10 stimulation of LH secretion has also been found in dispersed pituitary cells from pig and cows (Suzuki et al. 2007). In contrast, other studies have failed to find any effect of kisspeptins on the pituitary. Anterior pituitary fragments from adult male rats did not respond to Kisspeptin-10 but released LH and FSH

after stimulation with GnRH (Thompson et al. 2004). In another study, primary cultures of female rat anterior pituitary cells did not respond to human Kisspeptin-54 but released both FSH and LH when given GnRH with no potentiation of secretion using both peptides together (Matsui et al. 2004). A comparison of these studies is difficult because of differences in the experimental design but one variable is the time point at which the media was sampled for LH measurement. In the experiments of Navarro et al. (2005), the media was collected after 1 h and 3 h, while Thomson et al. (2004) collected media after 4 h. At the longer time points, the amount of LH in the media of the vehicle control group had increased which may have masked small kisspeptin effects. Alternatively, difficulties in measuring quite modest LH secretion may account for the differences.

Kisspeptin-10 has also been reported to stimulate modest growth hormone (GH) release from pituitary somatotrophs (Gutierrez-Pascual et al. 2007). In these studies, Kisspeptin-10 stimulated GH secretion from dispersed pituitary cells from peripubertal male and female rats but these responses were 20-fold less potent compared to that with the normal agonist GHRH. Kisspeptin-10 also stimulated a rise in cytoplasmic Ca^{+} levels in 60% of somatotrophs in the culture. The mechanism of this effect or its physiological significance is not clear. Transgenic mice with null mutations in the GPR54 receptor or the *Kiss1* gene do not have a major growth defect. In these culture experiments, GH secretion is being monitored in a heterogenous population of dispersed pituitary cells. It will be important to determine whether kisspeptins have a direct action on somatotrophs or whether the response is indirect and mediated by kisspeptin action on other cell types in the culture.

6.2 Metabolism

Kisspeptins may also act to integrate the peripheral signals of metabolic status to the central nervous system control of reproductive function. It has been noted that the age of menarche directly correlates with attainment of a minimum body fat composition. It has been suggested that the hormone leptin, a 16-kDa protein produced by adipose tissue, acts as a facilitator of puberty. In rodents, leptin can influence the onset of puberty although its role in primates is not clear. Leptin administration reduces the age at which puberty occurs in rats and mice (Ahima et al. 1997; Carro et al. 1997; Chehab et al. 1997) and female mice with mutations in the leptin gene (ob/ob) are sterile (Chehab et al. 1996). Administration of leptin to ob/ob mice can restore fertility (Mounzih et al. 1997). Similarly, mice with mutations in the leptin receptor (db/db) are sterile and fail to release GnRH (Coleman 1978; Johnson and Sidman 1979).

It has recently been shown that experimentally manipulated changes in the body energy status of rats can alter kisspeptin signalling in the hypothala-

mus. Fasting of prepubertal rats of either sex for 72 h, which is associated with a reduction in peripheral gonadotropin levels, causes a decrease in *Kiss1* and an increase in *Gpr54* in the whole hypothalamus as measured by RT-PCR (Castellano et al. 2005). Fasted animals still responded to central injection of Kisspeptin-10 by release of LH and these responses were larger than in non-fasted animals, perhaps due to the increase in GPR54. In a 30% food restriction model, which prevents puberty (measured by vaginal opening) in peripubertal rats, central injection of Kisspeptin-10 induced puberty in 60% of the animals (Castellano et al. 2005). Interestingly, leptin deficient ob/ob mice have decreased expression of *Kiss1* in the ARC which was increased by intraperitoneal injections of leptin over a period of four days (Smith et al. 2006a). Similar data have been found by Luque et al. (2007) where *Kiss1* and *Gpr54* mRNA levels increased in the whole hypothalamus in ob/ob mice when responses to reduced food intake were taken into account (Luque et al. 2007). These data may represent a direct action of leptin on *Kiss1* neurons as around 40% of *Kiss1* neurons expressed the leptin receptor (Smith et al. 2006a). Neuropeptide Y (NPY) null mice have reduced *Kiss1* expression in the hypothalamus which can be increased by NPY administration (Luque et al. 2007). It will be informative to relate these changes in *Kiss1* expression in the whole hypothalamus to specific changes in *Kiss1* in the AVPV and ARC regions. Nevertheless, these experiments demonstrate an interaction between energy status and the hypothalamic *Kiss1* system.

Kisspeptins may be involved in other metabolic disorders that affect reproductive function. Castellano and colleagues (2006) have studied the role of *Kiss1* in a rat model of type I diabetes that mimics the hypogonadotropic hypogonadism often found in uncontrolled diabetes (Castellano et al. 2006c). In the rat model, the alkylating reagent streptozotocin (STZ) is given to animals which selectively destroys the insulin producing β cells of the pancreas. STZ diabetic rats show a reduction in body weight, very low leptin levels, and defects in gonadotropin release and fertility (Steger et al. 1989) but retain GnRH production in the hypothalamus (Steger et al. 1989) and pituitary gonadotroph responses suggesting a defect upstream of GnRH secretion (Dong et al. 1991). STZ diabetic male rats have reduced *Kiss1* transcripts in the hypothalamus that increase after continuous intracerebral delivery of leptin but not insulin. These diabetic rats retain LH and testosterone secretory responses to ICV injection of Kisspeptin-10. Thus, the hypogonadotropic hypogonadism in this diabetic rat model may be caused by a leptin-related reduction in *Kiss1* expression reducing stimulation of the HPG axis (Castellano et al. 2006c).

Kisspeptins may also act at sites outside the neuroendocrine system. *Gpr54* expression has been detected by RT-PCR in cell lines of both pancreatic α and β-cell origin, while *Kiss1* expression was confined to the latter (Hauge-Evans et al. 2006). In the same study, kisspeptin and GPR54 were localized by immunohistochemistry to α and β cells of mouse pancreatic islets although

the specificity of the antibodies used should be considered. It was also shown that exogenous kisspeptin can enhance glucose-induced insulin secretion, but not glucagon secretion, from purified human or mouse islets while kisspeptin alone had no effect. Co-expression of kisspeptins and GPR54 in islets would allow paracrine or autocrine interactions but how these affect islet responses to glucose metabolism in vivo remains to be determined.

6.3 Ovary/Testes

Gpr54 and *Kiss1* are both expressed in the gonads but it is not known if this has a physiological role in spermatogenesis or ovary function. The cell types that express *Gpr54* in the testes have not been identified. Preliminary examination of heterozygous mice for β-galactosidase expression from the targeted *Gpr54* locus suggests that expression is confined to within seminiferous tubules but we have also detected *Gpr54* expression by RT-PCR in the Leydig cell line, MA-10 (unpublished). A direct action of kisspeptins on the testes is suggested in some studies by the inconsistent relationship between LH and testosterone release after systemic kisspeptin delivery. For example, Thompson et al. (2006) found that subcutaneous delivery of 50 nmol of Kisspeptin-14 in rats produced a very small increase in LH but gave a significant increase in total testosterone levels (Thompson et al. 2006). Similarly, continuous intravenous administration of Kisspeptin-10 in adult male rhesus monkeys gave more testosterone release when normalized to LH responses at higher kisspeptin doses (Ramaswamy et al. 2007). Thus, kisspeptins may enhance the effects of LH on stimulating testosterone production from the testes.

Kiss1 and *Gpr54* mRNAs are expressed in the rat ovary, and *Kiss1* shows a variation in expression during the estrus cycle with a 5-fold increase at proestrus (Castellano et al. 2006a). This increase in *Kiss1* expression correlated with the preovulatory LH surge as blockade of the LH surge prevented this increase which could be restored by injection of LH. Kisspeptin immunoreactivity was detected in the thecal layers of the growing follicles and in the corpora lutea, particularly the areas derived from the invading thecal cells. Thus, it is possible that *Kiss1* expression in the ovary might act as a local regulator of ovulation but is not essential for this as *Gpr54* null mice and a female patient homozygous for a GPR54 mutation (L148S) retain the ability to ovulate (Seminara et al. 2003; Pallais et al. 2006).

6.4 Placenta

Kiss1 is highly expressed by the human placenta and the level of kisspeptins increases at the 8th week of pregnancy from around 1 fmol/ml to more than 2000 fmol/ml for the remainder of the pregnancy (Horikoshi et al. 2003).

The significance of this dramatic rise is not clear, but this level of circulating kisspeptins would be expected to cause agonist suppression of GnRH release from the hypothalamus and shut down of the HPG axis as shown in rats (Thompson et al. 2006) and rhesus monkeys (Seminara et al. 2006). In the human placenta, kisspeptins are expressed only by syncytiotrophoblast cells (Bilban et al. 2004; Dhar et al. 2004; Horikoshi et al. 2003), which represent the cellular interface between the placenta and the maternal blood. In contrast, GPR54 expression is more extensive, being found in syncytiotrophoblast, villous and extravillous cytotrophoblast cells (Bilban et al. 2004). Expression of GPR54 by the highly invasive extravillous cytotrophoblast cells and the anti-metastatic activity of kisspeptins has led to the suggestion that these proteins may control placental invasion but the birth of *Gpr54* knock out mice and patients with homozygous mutations in GPR54 indicates that placentation can take place in the absence of fetal GPR54. Moreover, a female patient with a homozygous mutation in GPR54 (L148S) is reported to have conceived after hormonal treatment and given birth to a healthy child indicating that placentation can also take place in the absence of a functional maternal GPR54 (Pallais et al. 2006). What has not yet been established however, is the effect of loss of GPR54 from both the fetal and maternal placental compartments.

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