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The role of kisspeptin in the control of gonadotrophin secretion

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TABLE OF CONTENTS

BACKGROUND: Kisspeptins, and their cognate receptor gpr-54, were first found to regulate the hypothalamic–pituitary–gonadal (HPG) axis in 2003, when two groups demonstrated that mutations in gpr-54 cause idiopathic hypogonadotropic hypogonadism characterized by delayed or absent puberty. This review aims to highlight discoveries in the KiSS-1/gpr-54 system, focusing on their regulation of the HPG axis in male and female reproductive systems of both mammalian and non-mammalian vertebrates.

METHODS: A search of PUBMED and the authors' files was done without limitations by language or species for citations relevant to kisspeptin, reproduction and signal transduction.

RESULTS: Kisspeptins and gpr-54 are critical for puberty and the regulation of reproduction. Kisspeptins have been implicated in mediating many of the important signals relayed to the gonadotrophin-releasing hormone (GnRH) neuron such as positive and negative feedback, metabolic input and photoperiod. The ability of kisspeptin neurons to co-ordinate different signals impinging on the HPG axis makes it one of the most important regulators of GnRH and the reproductive axis.

CONCLUSIONS: Kisspeptins are pivotal regulators of the HPG axis and reproduction, with the ability to integrate signals from both internal and external sources. Knowledge about the signalling mechanisms involved in kisspeptin stimulation of GnRH would help improve the understanding of the importance of this critical pathway in reproduction.

Key words: kisspeptin / gpr-54 / gonadotrophins / steroid feedback / puberty

Introduction

Kisspeptins are peptide products of the KiSS-1 gene, which was first discovered by Lee *et al.* (1996) as a metastasis suppressing gene in malignant melanoma cells. As it was discovered in Hershey, it was decided to name the gene after Hershey's famous chocolate kisses and it has been whetting researchers' appetites ever since. The KiSS-1 gene is located on human chromosome 1q32 and consists of two non-translated and two partially translated regions and four exons to give rise to a 145 amino acid precursor peptide (West *et al.*, 1998). This precursor is then cleaved to 54 amino acids in length, which can be further truncated to 14, 13 or 10 amino acid carboxyl-terminal fragments (Fig. 1). Collectively, these N-terminally truncated peptides are known as the kisspeptins, and belong to a large family of peptides known as RFamides which all share a common Arg–Phe–NH_2 motif at their C-terminus. Kisspeptins are highly conserved within mammalian (Clements *et al.*, 2001; Kotani *et al.*, 2001) and non-mammalian vertebrates (Biran *et al.*, 2008; Kanda *et al.*, 2008; van Aerle *et al.*, 2008), suggesting they have an important role to play in a wide range of vertebrates.

Kisspeptins are the natural ligands for the orphan G protein-coupled receptor (GPCR) known as gpr-54 in rat (Lee *et al.*, 1999) and

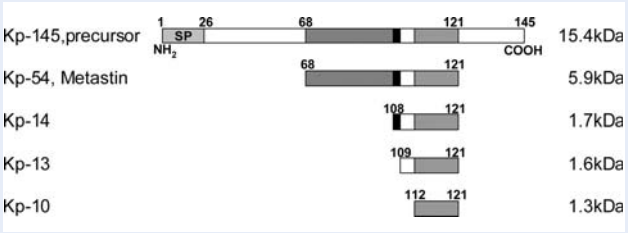

Figure 1 Schematic diagram showing the relative size and cleavage points for truncation of kisspeptin-145 (Kp-145) to the carboxyl-terminal fragments, Kp-54, Kp-14, Kp-13 and Kp-10. Also shown is the signal peptide (SP) within the Kp-145 precursor peptide.

AXOR12 in humans (Muir *et al*., 2001). However, for ease, throughout the rest of the review only gpr-54 will be used. Gpr-54 is a 396 amino acid receptor and is a member of the rhodopsin family/Class A GPCRs. It is most related to galanin receptors which have 45% homology; however, it has low affinity for galanin. Gpr-54 is highly conserved among mammals with the human receptor having 85% homology to the rat receptor, 80% homology to the mouse (Clements *et al*., 2001) and ~40% homology to non-mammalian vertebrates, such as bullfrogs (Moon *et al*., 2008). The 10 amino acid carboxyl-terminal sequence [kisspeptin-10 (Kp-10)] is the minimal length required for activation of gpr-54. The receptor couples to the $G_{q/11}$ class of G-proteins to activate phospholipase C which hydrolyses phosphatidyl bisphosphate in the cell membrane to diacyl glycerol which activates protein kinase C and inositol triphosphate, which modulates intracellular calcium. This leads to phosphorylation of ERK1/2 and p38MAPK, cellular reorganization of stress fibres and induction of focal adhesion kinase to inhibit cell movement, which is thought to be important for their inhibition of cancer metastasis (Hori *et al*., 2001; Kotani *et al*., 2001).

Although kisspeptins and gpr-54 were first described in relation to cancer metastasis, they were subsequently shown to play a pivotal role in the control of the hypothalamic–pituitary–gonadal (HPG) axis via regulation of gonadotrophin-releasing hormone (GnRH) secretion (Funes *et al*., 2003; Gottsch *et al*., 2004; Irwig *et al*., 2004; Messager *et al*., 2005). Immunohistochemical localization and mRNA analysis of the receptor and ligand within the brain and peripheral tissues, demonstrated gpr-54 and KiSS-1 within the hypothalamus, brainstem, spinal cord, pituitary, ovary, prostate and placenta. This localization suggests that they may regulate the reproductive axis at a number of levels (Clements *et al*., 2001; Kotani *et al*., 2001; Muir *et al*., 2001; Castellano *et al*., 2006a; Mead *et al*., 2006).

The discovery that kisspeptins are major neuroendocrine regulators of reproduction set the scene for revolutionary advances in unravelling the complexities of central, peripheral and exogenous regulation of reproductive neuroendocrinology. Here, we focus on kisspeptin regulation of the HPG axis, specifically on its role in the onset of puberty, in the release of GnRH, and in negative and positive feedback, as well as examining which other factors may regulate the kisspeptin/gpr-54 system.

Materials and Methods

An extensive search of PUBMED and the authors' files was done without limitations by language or species for citations relevant to kisspeptin, gpr-54, metastin and KiSS-1 in relation to the HPG axis. Citations from 1996 to present were included. A wider search of citations relevant to kisspeptin, reproduction and signal transduction was also performed without limitations of language, species or date, to broaden the background to this review. All papers that matched the search criteria and were relevant to this review were included. Diagrams were made using powerpoint, collating data from references within the review and the table was generated in excel, by the same means.

Kisspeptin is a key regulator of puberty and the HPG axis

Hypogonadotropic hypogonadism as a result of gpr-54 inactivating mutations

KiSS-1 was first shown to play a role in the reproductive axis in 2003, when two groups discovered that mutations in the gpr-54 receptor lead to idiopathic hypogonadotropic hypogonadism (iHH) in certain patients (de Roux *et al*., 2003; Seminara *et al*., 2003). In iHH, there is a deficiency of gonadotrophin secretion from the pituitary, as a result of impaired pubertal development of the HPG axis, usually due to mutations in the GnRH receptor. De Roux's group found that in five affected siblings, from first cousin marriages with normal GnRH receptor mRNA, a 155 nucleotide deletion in the gpr-54 receptor was the cause of their impaired puberty. This deletion did not cause impaired migration of GnRH neurons from the olfactory bulb, so it could not be classified as Kallmann's syndrome, but was classified as iHH and is therefore due to disrupted stimulation of GnRH production (de Roux *et al*., 2003). Seminara *et al*. found a separate gpr-54 mutation, L148S, in another family with six affected members also from first cousin marriages. This mutation caused impaired signalling of the gpr-54 receptor resulting in iHH. In the same study, a non-related male with iHH was also found to have gpr-54 mutations, R331X and X339R, causing elongation of the receptor sequence due to a disrupted stop codon (Seminara *et al*., 2003). Discovery of these mutations identified gpr-54 and kisspeptins as important regulators of puberty and the HPG axis. Since 2003, further mutations of the gpr-54 receptor have been found to cause iHH in humans. Semple *et al*. (2005) found C223R and R297L mutations in one male patient and these gave rise to impaired signalling, with C223R causing a more severe phenotype than R297L in the impairment of kisspeptin stimulation of calcium release *in vitro*. Another group reported that mutation 1001–1002insC caused iHH in association with cryptorchidism (Lanfranco *et al*., 2005). Then in 2007, five iHH patients were shown to have a L102P mutation in gpr-54 (Tenenbaum-Rakover *et al*., 2007). However, gpr-54 mutations only account for ~2% of iHH cases (Pedersen-White *et al*., 2008) and all can be rescued by treating with GnRH or gonadotrophins, which induce puberty and normal reproductive function, allowing an affected individual to lead a normal reproductive life (Table I).

Precocious puberty as a result of gpr-54 activating mutations

As well as inactivating mutations of the gpr-54 receptor, an activating mutation, R386P, caused prolonged signalling of the receptor leading to precocious puberty in a girl of 8 years of age (Teles *et al*., 2008). A polymorphism prevalent in Chinese girls with precocious puberty, P110T, was also found to be statistically associated with the condition, although further evidence is needed to confirm that this substitution causes receptor activation (Luan *et al*., 2007).

KiSS-1 and gpr-54 knockout mice display hypogonadotropic hypogonadism characteristics

At the same time as gpr-54 mutations were being found to cause iHH in humans, a gpr-54 knockout mouse had been produced with similar

Table I Table summarizing all inactivating and activating mutations of gpr-54 reported to date

Mutation	Phenotype	Receptor impairment
155 nuc. deletion	iHH	Truncation of receptor
L148S	iHH	Impaired signalling
R331X & X339R	iHH	Receptor elongation
C223R	iHH	Impaired signalling
R297L	iHH	Impaired signalling
L102P	iHH	Impaired signalling
1001ins1002C	iHH and cryptorchidism	Receptor elongation
R386P	Precocious puberty	Prolonged signalling
P110T	Precocious puberty	Not known

The table shows the mutation, phenotype and effect the mutation has on receptor sequence or signalling. References are shown in the text.

findings to those from the patients described above. Gpr-54−/− male mice had small testes, delayed puberty and reduced sexual behaviour and females had small ovaries and uteri, delayed vaginal opening, no maturation of follicles in the ovary and reduced sexual behaviour. Both sexes were infertile and had low gonadotrophin levels although low magnitude pulses could still be detected, suggesting that gpr-54 signalling is not required for basal GnRH or luteinizing hormone (LH) levels in the mouse. Gpr-54−/− mice are still responsive to exogenous GnRH, showing that there is an impaired secretion of GnRH in these animals (Seminara *et al*., 2003; Lapatto *et al*., 2007). The kisspeptin system, therefore, appears to elevate GnRH pulse amplitude more than frequency (Seminara *et al*., 2003). These results confirm the human observations and place kisspeptins and gpr-54 as major regulators of puberty.

More recently, KiSS-1 knockout mouse models have been developed that also display iHH characteristics; however, the physiological effects seem more variable than in the gpr-54 knockout. In one study, although infertile, only 50% of females had smaller uteri and ovaries with 50% being normal; however, all had reductions in gonadotrophins and sexual behaviour (Lapatto *et al*., 2007). In a different study, all KiSS-1−/− mice were infertile with delayed puberty. Female mice had delayed vaginal opening, small ovaries, thread-like uteri and no estrus cycle or follicle maturation due to decreased gonadotrophin secretion. The male mice also had delayed pubertal maturation, small testes, microphallus, spermatogenic arrest and decreased gonadotrophins and sex steroids (d'Anglemont de Tassigny *et al*., 2007). This shows the variability of the phenotype, as in one study KiSS-1 knockout seems to be partially compensated for, but in the other the KiSS-1 knockout cannot be overcome by compensation. However, both sets of animals were responsive to exogenous kisspeptin, showing that both active kisspeptin and gpr-54 are required for puberty to occur at the required time. The less complete phenotype in the KiSS-1 knockout mice than gpr-54 knockout mice suggests that other RFamide peptides may be able to partially compensate for kisspeptin and activate gpr-54.

KiSS-1 mRNA and gpr-54 sensitivity in the hypothalamus increases with pubertal development

The realization of the pivotal role of the KiSS-1/gpr-54 system stimulated further research in the quest to elucidate the mechanistic details. It has been demonstrated that KiSS-1 and possibly gpr-54 mRNA hypothalamic levels increase with pubertal maturation in mice (Han *et al*., 2005), rats (Sun *et al*., 2007) and primates (Shahab *et al*., 2005; Keen *et al*., 2008). In rodents, KiSS-1 mRNA increases in the anteroventral periventricular nucleus (AVPV) but not in the arcuate nucleus (Arc) during puberty. There is also an increase in the number of GnRH neurons that are depolarized by kisspeptin during puberty. In juvenile mice, only 27% of GnRH neurons depolarize with exogenous kisspeptin treatment, this increases to 45% in prepubertal mice and 90% in the adult. This increase in the number of GnRH neurons that are depolarized appears to be due to the increase in gpr-54 sensitivity at this time and the increased kisspeptin neuron apposition with GnRH neurons (Han *et al*., 2005), along with the increase in kisspeptin mRNA levels, which is shown to occur throughout puberty. It has been reported in the mouse, that kisspeptin neuron's close association with GnRH neurons in the preoptic area (POA) and median eminence (ME) increase with puberty, appearing from post-natal day (P) 25 and reaching adult like proportions of 50% co-localization in the POA at around P61 (Clarkson and Herbison, 2006). As mentioned above, another explanation for the increase in depolarization is that GnRH neurons and gpr-54 gain sensitivity to kisspeptin during puberty in the mouse. Additionally, in primates, KiSS-1 mRNA increases during puberty at the same rate as GnRH mRNA. In prepubertal female rhesus monkeys, KiSS-1 mRNA increases nocturnally then by mid-puberty KiSS-1 and gpr-54 mRNA increase 3-fold in the Arc and kisspeptin secretion in the ME becomes pulsatile with 60 min intervals. Of these pulses, 75% correlate with GnRH pulses (Shahab *et al*., 2005; Keen *et al*., 2008). These pulses then continue into adulthood to regulate the HPG axis. In males, KiSS-1 mRNA increases with puberty but gpr-54 mRNA does not, suggesting that increasing KiSS-1 mRNA is a controlling factor in males.

In order to look at the physiological consequences of increased kisspeptin during puberty, studies were undertaken to look at the effect of repetitive administration of exogenous kisspeptin to juvenile female rats (Navarro *et al*., 2004b) and primates (Plant *et al*., 2006). This would reveal whether kisspeptin alone is sufficient to kick start the onset of puberty. In both studies, repetitive kisspeptin administration was able to advance puberty, measured in the mouse as advanced vaginal opening and increased uterine weight and in both species gonadotrophins were elevated to adult levels (Navarro *et al*., 2004b; Plant *et al*., 2006). This was revealed in both cases to be due to kisspeptin stimulation of GnRH secretion, as GnRH antagonists completely abolished the advancement of puberty.

The evidence above suggests that kisspeptin and gpr-54 are key regulators of puberty in mammals due to a programmed increase in KiSS-1 mRNA and increased gpr-54 sensitivity to kisspeptin, possibly due to an increase in receptors at the cell surface. Activation of the kisspeptin system facilitates increased pulsatile release of GnRH, awakening the reproductive axis and bringing about pubertal maturation.

Kisspeptin/gpr-54 regulate the HPG axis through modulation of GnRH, LH and follicle stimulating hormone

Acute kisspeptin stimulates secretion of LH and follicle stimulating hormone through GnRH

As mentioned above, the expression of both ligand and receptor in the hypothalamus and pituitary and their crucial role in puberty suggest that kisspeptins may also be key regulators of the HPG axis in adults. In the gpr-54−/− and KiSS-1−/− mice, LH levels were found to be significantly lower than in WT mice even though GnRH receptor levels were normal and GnRH could elicit a robust LH stimulation. This indicated a role for kisspeptins in LH regulation in the adult. Kp-54 and Kp-10 rapidly increase plasma LH levels in adult mice (Gottsch *et al*., 2004),

rats (Navarro *et al*., 2004a), sheep (Arreguin-Arevalo *et al*., 2006), cows (Kadokawa *et al*., 2008), primates (Plant *et al*., 2006) and humans (Dhillo *et al*., 2005, 2007) in a dose dependant manner. Doses as low as 1 fmol are effective (Navarro *et al*., 2005b), making kisspeptin the most potent stimulator of LH known to date. Low doses have been shown to be active both centrally (i.c.v.) and systemically (i.p. and i.v.) at similar levels in rodents (Thompson *et al*., 2004; Navarro *et al*., 2005b). The demonstration that kisspeptin stimulation of gonadotrophins can be abolished by administering a GnRH receptor antagonist suggests that kisspeptin operates at the level of the hypothalamus to stimulate GnRH release rather than directly at the pituitary (Gottsch *et al*., 2004; Navarro *et al*., 2005b). Castellano *et al*. have attempted to delineate the mechanism behind kisspeptin activation of gonadotrophins in the rat. Kisspeptin stimulation of LH could be blocked by PLC, calcium and MAPK inhibitors using hypothalamic explants pre-incubated for 60 min, however, no further evidence to support these pathways has been produced (Castellano *et al*., 2006b).

In gpr-54 and KiSS-1 knockout mice, follicle stimulating hormone (FSH) levels also are lower than in controls (Lapatto *et al*., 2007). Again, Kp-10 has been shown to stimulate release of FSH in adult rats (Navarro *et al*., 2005a), mice (Gottsch *et al*., 2004) and humans (Dhillo *et al*., 2005). However, in the rat a 100-fold higher dose is required to stimulate FSH than for LH secretion, with an EC50 of 400 nmol. Activation of FSH secretion is also through GnRH, as GnRH antagonist again blocks the Kp-10 stimulation (Navarro *et al*., 2005a). However, most studies have concentrated research on LH because FSH stimulation is of a much lower magnitude than that of LH. Further studies are required to fully elucidate kisspeptins effects on FSH.

Chronic kisspeptin administration disrupts secretion of LH and FSH

As shown above acute injection of kisspeptin stimulates LH release and repeated injections produce LH pulses (Tovar *et al*., 2006). Continuous i.v. injections of kisspeptin in rodents and primates over 4 days initially stimulates LH, but after 3–4 h stimulation this rise begins to fall reaching control levels by 24 h. This has been attributed to desensitization of the gpr-54 receptor, as GnRH injection is still able to increase LH secretion during the last 3 days of kisspeptin infusion in adult male mice and primates (Ramaswamy *et al*., 2007; d'Anglemont de Tassigny *et al*., 2008). Also, after the infusion is completed, acute kisspeptin administration cannot induce LH until the receptor recovers ~2 h later. However, in female adult rats, the elevation of LH lasted for 48 h during a 7 day infusion and in peri-pubertal female rats, desensitization did not occur, suggesting a change in gpr-54 sensitivity during puberty or an increase in inputs to gpr-54 from kisspeptin neurons (Roa *et al*., 2008c). Nevertheless, it is evident that gpr-54 desensitizes with time, so chronic administration may be a useful tool therapeutically for inhibiting gonadotrophins and sex steroids as currently accomplished by GnRH agonists in treating hormone-dependant diseases.

Kisspeptin neurons are located in the hypothalamus and directly contact GnRH neurons

Kisspeptin mRNA and protein have been localized in the Arc and the AVPV of the rodent hypothalamus, with a small group of neurons also being identified in the periventricular nucleus (PeN; Gottsch *et al*., 2004; Clarkson and Herbison, 2006; Kauffman *et al*., 2007a). However, in the ewe and in primates, kisspeptin neurons are not located within the AVPV (Franceschini *et al*., 2006; Rometo *et al*., 2007). The kisspeptin neurons project into the ME and POA regions within the hypothalamus, which also possess GnRH neurons (Franceschini *et al*., 2006; Pompolo *et al*., 2006). Double immunoflourescence has revealed that 85% of the GnRH neurons in the ME of rats contain gpr-54 on their cell surface and express increased levels of c-fos in response to Kp-10 (Irwig *et al*., 2004; Matsui *et al*., 2004). There is also a close association between 70–90% of GnRH and kisspeptin neurons in mice, sheep and rhesus monkey, suggesting that kisspeptin acts directly at the GnRH neurons to regulate the HPG axis. However, these associations differ in brain regions between different species. In the mouse, kisspeptin axons associate with GnRH cell bodies and dendrites in the POA (Clarkson and Herbison, 2006). In the sheep, co-localization is seen in both the ME and POA (Pompolo *et al*., 2006), and in the primate, associations occur in the ME but this time between Kisspeptin and GnRH axons (Ramaswamy *et al*., 2008). However, it is yet to be determined if these differences are species related or simply due to differences in the primary and secondary fluorescent antibodies used in each study.

Kisspeptin and gpr-54 are present in the anterior pituitary

The role of kisspeptin in the pituitary has been debated, with conflicting results obtained *in vitro* on primary pituitary cell cultures. Some groups have seen no effect of kisspeptin on rat anterior pituitary cells (Matsui *et al*., 2004), whereas another group showed increased Ca^{2+} in 10% of cells, along with stimulation of LH and growth hormone (GH; Gutierrez-Pascual *et al*., 2007). However, the doses used in this study were extremely high compared with other studies and the effects may be due to activation of other RFamide receptors. LHβ expressing rat pituitary cells express KiSS-1 mRNA; however, KiSS-1 mRNA was also detected in other pituitary cells, possibly somatotropes. Gpr-54 mRNA was located in a subset of LHβ expressing cells, suggesting the machinery is present for kisspeptin to have direct pituitary effects. Pituitary KiSS-1 mRNA appears to be regulated by estrogen and gpr-54 appears to be regulated by GnRH, making it plausible for direct effects to occur in the rat (Richard *et al*., 2008). However, all of these studies have used highly sensitive PCR techniques that do not reveal a functionally significant expression of gpr-54 and kisspeptin. Moreover, pharmacological doses of kisspeptin were required to elicit effects on the pituitary.

In ovine anterior pituitary cells, gpr-54 mRNA is present in gonadotropes, somatotropes and lactotropes. Administering kisspeptin to these cells when taken during the follicular phase causes an 80% increase in LH release. However, in ovariectomized (OVX) ewes which were hypothalamic–pituitary disconnected (hence ablating effects of GnRH on the pituitary), no stimulation of LH by kisspeptin was seen at any time during the cycle. Furthermore, although kisspeptin is present at low levels in the hypophysial blood, this was not affected by estrogen, even at LH surge levels (Smith *et al*., 2008). Thus, *in vivo* findings in contrast to *in vitro* findings indicate that direct pituitary effects of kisspeptin are not important in LH release. Overall, there is no convincing data demonstrating that direct effects of kisspeptin at the level of the pituitary is important for regulation of the HPG axis.

Kisspeptin and gpr-54 mediate steroid feedback on the HPG axis

Kisspeptin and gpr-54 mediate negative steroid feedback within the HPG axis

It has been known for many years that the HPG axis is under the control of steroid hormone feedback from the gonads. Steroid hormone levels fluctuate across the cycle in females (Almond and Dial, 1990; Karsch and Evans, 1996; Gill *et al*., 2002; Moenter *et al*., 2003). However, the mediator of steroid hormone feedback has remained elusive for many years, as GnRH neurons only possess estrogen receptor β (ERβ) that does not play a role in feedback. Therefore, neurons upstream of the GnRH neuron, which possess estrogen receptor α (ERα), progesterone receptor (PR) and androgen receptor (AR) have been sought as possible mediators of steroid effects on GnRH release.

Kisspeptin neurons express ERα, PR and AR and therefore have the potential to relay feedback effects on the GnRH neuron. Regulation of KiSS-1 expression is likely to be a mediator of negative feedback in mouse (Smith *et al*., 2005a, b), rat (Adachi *et al*., 2007), ewe (Franceschini *et al*., 2006; Smith *et al*., 2006b; Goodman *et al*., 2007) and human (Rance, 2008). Evidence now suggests that reduced activity of kisspeptin neurons in the Arc of rodents, primates and sheep is responsible for translating estrogen negative feedback to GnRH neurons. OVX female and castrated male mice (Smith *et al*., 2005a, b), sheep (Pompolo *et al*., 2006; Smith *et al*., 2006b) and rhesus monkeys (Rometo *et al*., 2007; Shibata *et al*., 2007) have an increased level of KiSS-1 mRNA in the neurons compared with controls. Also, if estrogen replacement is given to OVX female or testosterone is given to castrated male mice, sheep and rats, then KiSS-1 mRNA levels are reduced to control levels (Smith *et al*., 2005b, 2006b, c; Pompolo *et al*., 2006; Rometo *et al*., 2007; Shibata *et al*., 2007). This suggests that steroids are negatively regulating KiSS-1 mRNA in the Arc, hence reducing stimulation of GnRH neurons. KiSS-1 mRNA regulation by steroids has been demonstrated to be specifically through the ERα isoform as PPT, a specific inhibitor for this isoform blocks the reduction of neuronal mRNA in the Arc, but DPN an ERβ specific inhibitor does not effect these mRNA levels (Bateman and Patisaul, 2008). Also ERα−/− mice do not have any steroid negative feedback on KiSS-1 mRNA levels in the hypothalamus (Smith *et al*., 2005a).

This negative regulation of kisspeptin by steroids was also revealed by KiSS-1 mRNA regulation across the rat's ovarian cycle in the Arc, where levels increased at diestrus when estrogen levels are low and decreased at pro-estrus when estrogen levels are elevated in rats (Adachi *et al*., 2007). Further evidence for negative feedback is derived from females at the time of menopause, when estrogen is low due to reduced follicle numbers in humans and rhesus monkeys. At this time, a rise in KiSS-1 mRNA and in turn LH is seen accompanied by cellular hypertrophy, similar to the rise in OVX female rhesus monkey (Rometo *et al*., 2007; Kim *et al*., 2008; Rance, 2008), which is thought to be due to the lack of negative feedback from the follicles.

However, kisspeptin cannot be the only factor effecting GnRH secretion, since in the gpr-54 knockout mouse, there are still basal pulses of LH (Seminara *et al*., 2003). It has been shown that KiSS-1 co-localizes with two other ERα, PR and AR positive peptidergic neurons; the dynorphin A neuron and the neurokinin B neuron. It is possible that they work in concert to regulate negative feedback in the Arc (Goodman *et al*., 2007).

Kisspeptin and gpr-54 mediate positive steroid feedback

As well as regulating negative feedback, estrogen also regulates positive feedback on the HPG axis at the time of the LH surge in females. The LH surge occurs when estrogen reaches a threshold level synchronizing GnRH neuron secretary activity to stimulate the LH surge for ovulation, followed by a secondary FSH surge on the morning of estrus in rats (Roa *et al*., 2008a). Again, due to the lack of ERα in GnRH neurons, estrogen appears to act upstream of these neurons. Kisspeptin neurons are perfectly placed to mediate this response. In mice and rats, positive feedback is regulated by a separate group of kisspeptin neurons in the AVPV, which are associated with the LH surge (Smith *et al*., 2005a, 2006d; Adachi *et al*., 2007). This association can again be demonstrated at the level of the neuron, where KiSS-1 mRNA is decreased in the AVPV in OVX female rats and its levels increase with estrogen replacement (Smith *et al*., 2005a). Positive feedback on KiSS-1 mRNA in the AVPV can also be shown in intact female rats, where AVPV KiSS-1 mRNA increases on the afternoon of pro-estrus and expression of c-fos increases just before the LH surge when estrogen is at its highest levels (Adachi *et al*., 2007). The kisspeptin neurons in the AVPV project to the POA and if kisspeptin is blocked in the POA then the LH surge cannot occur, proving the importance of kisspeptin neurons in positive feedback (Kinoshita *et al*., 2005). As with negative feedback, positive feedback is regulated through ERα as blockade of this receptor completely inhibits the gonadotrophin surge and ovulation in mice and rats (Kinoshita *et al*., 2005; Adachi *et al*., 2007; Roa *et al*., 2008b).

In contrast to mice and rats, in the sheep and primates kisspeptin neurons are only localized to the Arc and POA. Here, the Arc appears to be responsible for both negative and positive feedback (Estrada *et al*., 2006; Pompolo *et al*., 2006; Smith *et al*., 2007). In adult ewe neurons, KiSS-1 mRNA in the caudal Arc rises during the follicular phase and in the rostal Arc at estrus when the LH surge occurs (Estrada *et al*., 2006). This suggests that the Arc is responsible for modulating the positive steroid feedback in the ewe. In the intact ewe, during the estrus cycle, kisspeptin can synchronize LH surges and during anoestrus, administration of kisspeptin can cause ovulation to occur, suggesting that when kisspeptin levels are high enough they can cause the LH surge and are therefore probably involved in relaying positive feedback to GnRH neurons (Caraty *et al*., 2007).

Steroids are responsible for sexual dimorphism of KiSS-1 expression in neurons in the AVPV

In the AVPV of rats, females have 12-fold higher KiSS-1 expression than males, probably in order to produce a synchronized LH surge. This elevation appears to be due to steroid exposure during neonatal life. If female rats are exposed to testosterone neonatally, they develop male levels of KiSS-1 in the AVPV and conversely male gpr-54 knockout mice develop female levels, due to low testosterone levels. However, KiSS-1 levels in the rat Arc are not affected (Kauffman *et al*., 2007a, b). This suggests that testosterone in neonatal life, in both males and females, is important in developmental differentiation of kisspeptin neurons.

Overall, it is apparent that kisspeptin neurons are important mediators of steroid feedback in many species, to regulate LH release throughout the cycle and to synchronize release at the time of the GnRH/LH surge (Fig. 2). Evidently, steroids are important regulators of kisspeptin neurons and play an important role in the regulation of the HPG axis in developmental, prepubertal and adult life.

KiSS-1 neurons are influenced by environmental and metabolic factors

Kisspeptin is regulated by photoperiod in seasonal breeders

Environmental factors such as photoperiod play a regulatory role in the HPG axis through regulation of GnRH production (Porkka-Heiskanen *et al*., 1997). In view of its major role in the regulation of the GnRH neuron, kisspeptin neurons were hypothesized to relay photoperiod effects on GnRH neurons in seasonally breeding species. In seasonal breeders, activation of the reproductive axis is controlled by melatonin produced from the pineal gland, which is secreted at night only and therefore relays day length to the body (Bittman *et al*., 1985). Three animal models have been employed to look at these effects, the Siberian and Syrian hamsters [long day (LD) breeders] and sheep [short day (SD) breeders].

Female Siberian hamsters are sexually active during LDs, when melatonin secretion is reduced. During this time, KiSS-1 mRNA in the Arc is decreased, although AVPV KiSS-1 mRNA is increased, to give optimum conditions for conception. However, during SDs when female Siberian hamsters are in anoestrus the opposite occurs, with high KiSS-1 levels in the Arc and low levels in the AVPV (Greives *et al*., 2006; Mason *et al*., 2007). In the Syrian Hamster, also a LD breeder, no staining was found in the AVPV, but an increase in KiSS-1 mRNA was seen in the Arc during LD and a decrease was seen during SD. This response to seasonal breeding appears to be a result of melatonin production as pineal gland

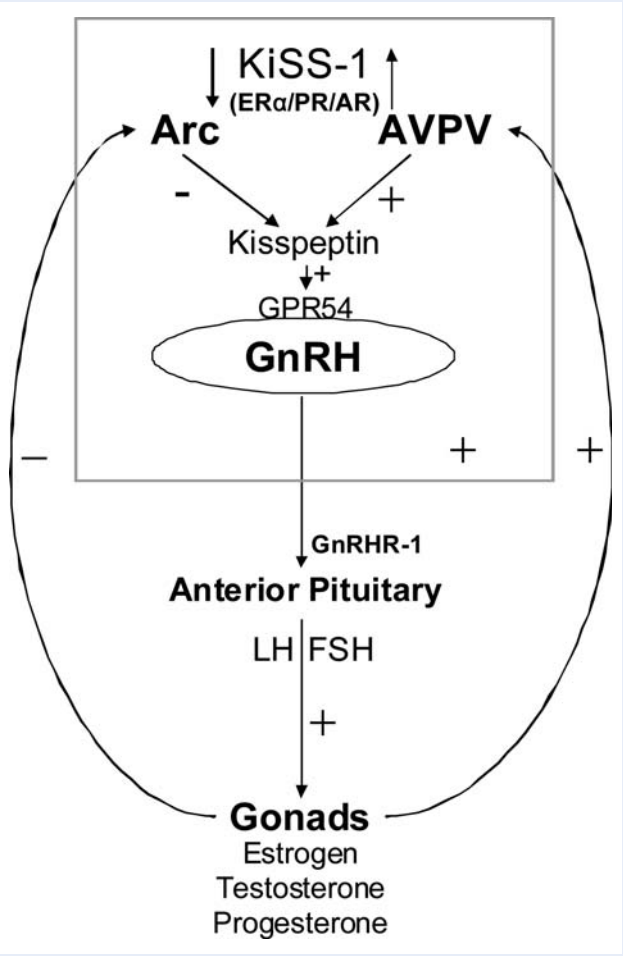

Figure 2 Diagram showing positive and negative feedback inputs into the hypothalamic-pituitary-gonadal (HPG) axis through KiSS-1 neurons in the anteroventral periventricular nucleus (AVPV) and arcuate nucleus (Arc) within the hypothalamus of rodents (grey box), which are projecting to co-localise with GnRH neurons in the preoptic area and median eminence.In sheep and primates, only the Arc is involved. ER, estrogen receptor; PR, progesterone receptor; AR, androgen receptor.

ablation prevents the reduction in Arc KiSS-1 during SD (Revel *et al*., 2006). Thus, melatonin is important for KiSS-1 regulation in both Siberian and Syrian hamsters but evidence of functional melatonin receptors on kisspeptin neuronal cells has not been demonstrated.

In the ewe, which is an SD breeder and so responds to a long melatonin secretion period, KiSS-1 mRNA in the Arc is increased during SD when estrus occurs, and Arc KiSS-1 expression is decreased during LDs for anoestrus (Smith *et al*., 2006b). This allows activation of the LH surge during estrus but inhibition during anoestrus (a mechanism which has evolved to co-ordinate reproduction with seasonal availability of food). However, seasonal endocrine physiology can be overcome with exogenous kisspeptin, which when given continuously for 30 h can cause ovulation in 80% of ewes during anoestrus (Caraty *et al*., 2007). This shows that kisspeptin is a potent regulator of the HPG axis, even when the system is dampened.

Therefore, kisspeptin neurons represent a conserved mechanism for relaying photoperiodic cues in seasonal breeding animals. The neuronal networks and melatonin actions are yet to be delineated.

Kisspeptin is regulated through leptin signalling

Metabolic factors, such as body mass index (BMI) have profound effects on reproduction. This is manifestly evident in female dancers and athletes when body weight declines to low levels during intense training to produce amenorrhea (Shade, 1983). Leptin, an adipose derived hormone is known to affect metabolism, food intake and reproduction (Bluher and Mantzoros, 2007). It has been reported that leptin increase is involved in the onset of puberty (Bluher and Mantzoros, 2007). Around 40% of kisspeptin neurons in the mouse possess leptin receptors (Smith *et al*., 2006a), this implicates kisspeptin neurons as a mediator of metabolic control of the HPG axis and puberty.

In order to examine the affect of leptin on KiSS-1, two models, the ob/ob mouse, which lacks leptin, and states of under-nutrition have been studied. In the ob/ob mouse, KiSS-1 expression in the arcuate nucleus is reduced compared with controls and this is partially rescued by leptin replacement (Smith *et al*., 2006a). The same is also true for gpr-54 mRNA, indicating that leptin can regulate KiSS-1 and gpr-54 mRNA (Luque *et al*., 2007). In adult mice subjected to short-term fasting, there is a rapid decline in both KiSS-1 and gpr-54 mRNA at around 12–24 h followed by a decrease in GnRH at 48 h (Luque *et al*., 2007). In fasting prepubertal rats, KiSS-1 is still decreased but gpr-54 is increased at this stage (Castellano *et al*., 2005). However, opposite gpr-54 responses are seen in underfed animals subjected to continuous kisspeptin infusion. In control adult rats, continuous kisspeptin infusion causes LH to rise for 48 h, which then drops to basal levels, suggesting receptor desensitization. In the presence of kisspeptin, in underfed female adult rats, the LH response is prolonged to 5 days and in underfed female peri-pubertal rats, LH responds for the whole 7 days with no sign of desensitization (Roa *et al*., 2008c). These results suggest that leptin positively regulates gpr-54 mRNA in pubertal and adult life, as these responses were mimicked by leptin administration, but gpr-54 appears to be more sensitive to leptin during puberty. The above evidence places kisspeptin and gpr-54 as main mediators of external and internal signals to GnRH and the HPG axis.

Kisspeptin and gpr-54 may have direct actions at the ovary

Kisspeptin expression and effects in the ovary

Expression of KiSS-1 and gpr-54 has been seen in peripheral tissues in addition to in the hypothalamus and brain, with the most abundant expression in the placenta. Another region where expression has been noted is within the testis and the ovary. No functional effect of kisspeptin has been found in the testis, but regulation of KiSS-1 mRNA around the time of ovulation has been noted in the ovary. In the rat ovary, KiSS-1 and gpr-54 mRNA expression is found in the ovarian surface epithelium and interstitial glands at all stages of the cycle. Whereas KiSS-1 and gpr-54 expression in the follicle was stage dependant, with staining in the theca layer of growing and pre-ovulatory follicles from estrus to early pro-estrus, which then moved to the granulosa cell layer of pre-ovulatory follicles in late pro-estrus. After ovulation, expression returned to the theca-lutein cells of the corpus luteum and expression decreased as the corpus luteum regressed. Also expression levels fluctuated with the estrus cycle with expression increasing on the afternoon of pro-estrus just preceding the LH surge and ovulation and then sharply decreasing thereafter. This increase in KiSS-1 mRNA seems to be directly associated with the increase in LH at the time of the surge. If the LH surge is absent then this rise does not occur. Administration of hCG, to these animals, increased KiSS-1 mRNA in the ovary, suggesting the ovarian rise in KiSS-1 mRNA is regulated by LH. In prepubertal female rats, KiSS-1 expression in the ovary is very low, due to low LH, but this can again be overcome by hCG administration (Castellano *et al*., 2006a). Therefore,

kisspeptin and gpr-54 show spatial and temporal expression changes in the ovary during the cycle, which suggests a functional role.

Conclusions

Kisspeptin is a key regulator and mediator of reproductive biology in a variety of mammals. In the female, kisspeptin is responsible for modulating the LH surge and ovulation as well as day to day release of GnRH. In this respect, there are two distinct sets of neurons in rodents (Roa *et al*., 2008b) and one dominant set in primates (Ramaswamy *et al*., 2008) and sheep (Smith *et al*., 2006b).

The importance of kisspeptin as a prime neuroendocrine regulator of reproduction is underlined by its high level of conservation between mammalian species. The kisspeptin/gpr-54 system also appears to be conserved in non-mammalian vertebrates including fish (Nocillado *et al*., 2006; Mohamed *et al*., 2007; Biran *et al*., 2008; Kanda *et al*., 2008; van Aerle *et al*., 2008), amphibians (Moon *et al*., 2008) and avian species.

The importance of kisspeptins in the HPG axis is also reflected by its tight regulation by internal and external factors. Steroids hormones regulate kisspeptin delivery to GnRH neurons in positive and negative modalities to synchronize the ovarian cycle. Leptin and melatonin regulate kisspeptin to synchronize reproduction with environmental conditions in seasonal breeders (Mason *et al*., 2007). This mechanism is critical for the species survival to ensure birth coincides with the occurrence of plentiful food and good environmental conditions to rear young. Also, the regulation of kisspeptin by leptin facilitates in relaying the existence of adequate energy stores before initiation of breeding. Leptin is involved in the onset of puberty, and turns off the reproductive system if energy stores are too low to cope with the demands of the HPG axis (Luque *et al*., 2007). The fact that kisspeptin, and not GnRH, is directly modulated by these factors places it in a critical position within the pathway.

More recently, it has become apparent that ovulation in the rat may be controlled by kisspeptin at the level of the ovary as well as the hypothalamus, with a pre-ovulatory rise occurring in the granulosa cells in response to the LH surge, to stringently control this critical mechanism (Castellano *et al*., 2006a).

In just 5 years, since the discovery of kisspeptin/gpr-54 as pivotal regulators of the HPG axis, impressive advances have been made in delineating the physiological role of this new frontier of reproductive neuroendocrinology. Nevertheless, there are substantial gaps in areas such as the molecular aspects of kisspeptin/gpr-54 interactions, intracellular signalling and the regulation of KiSS-1 and gpr-54 gene expression at an intracellular biology level. There is also a need to develop kisspeptin agonists and antagonists as pharmacological tools and as therapeutics for a range of clinical conditions.

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