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Anatomy of the kisspeptin neural network in mammals

Michael N. Lehman[a,b,*], **Christina M. Merkley**[a,b,*], **Lique M. Coolen**[a,b], and **Robert L. Goodman**[c]

[a] Department of Anatomy and Cell Biology, The University of Western Ontario, London, Ontario, Canada, N6A 5C1

[b] Neuroscience Graduate Program, The University of Western Ontario, London, Ontario, Canada, N6A 5C1

[c] Department of Physiology and Pharmacology, West Virginia University, Morgantown, West Virginia, United States of America, 26506

Abstract

Kisspeptin has been recognized as a key regulator of GnRH secretion during puberty and adulthood, conveying the feedback influence of endogenous gonadal steroids onto the GnRH system. Understanding the functional roles of this peptide depends on knowledge of the anatomical framework in which it acts, including the location of kisspeptin-expressing cells in the brain and their connections. In this paper, we review current data on the anatomy of the kisspeptin neuronal network, including its colocalization with gonadal steroid hormone receptors, anatomical sites of interaction with the GnRH system, and recent evidence of neurochemical heterogeneity among different kisspeptin neuronal populations. Evidence to date suggests that kisspeptin cells in mammals comprise an interconnected network, with reciprocal connections both within and between separate cell populations, and with GnRH neurons. At the same time, there is more functional and anatomical heterogeneity in this system than originally thought, and many unanswered questions remain concerning anatomical relationships of kisspeptin neurons with other neuroendocrine and neural systems in the brain.

Keywords

kisspeptin; hypothalamus; neuroanatomy; GnRH; steroid receptor

1. Introduction

Kisspeptin is one of a family of RFamide-related peptides (RFRP) that is now recognized as an essential endogenous regulator of the GnRH neuroendocrine system (Oakley et al., 2009). Kisspeptin and its related peptides are ligands for the orphan G protein-coupled receptor 54 (GPR54, now called Kiss1 receptor), mutations of which produce hypogonadotropic hypogonadism and a failure to enter puberty in humans (de Roux et al., 2003; Seminara et al., 2003) and mice (Seminara et al., 2003). Kisspeptin was subsequently shown to be an

Corresponding Author: Michael N. Lehman, Department of Anatomy and Cell Biology, University of Western Ontario, London, Ontario, Canada, N6A 5C1, Phone: 519-661-2111 ext. 84054; Fax: 519-661-3936, michael.lehman@schulich.uwo.ca.
*M. N. Lehman and C. M. Merkley contributed equally to this work

extraordinarily potent stimulator of GnRH/LH secretion in a variety of species (Irwig et al., 2004; Jayasena et al., 2009; Messager et al., 2005; Shahab et al., 2005), and because of the presence of Kiss1 receptors in GnRH neurons (Han et al., 2005; Irwig et al., 2004) and the ability of GnRH antagonists to block the effects of kisspeptin (Shahab et al., 2005), early work quickly suggested that this influence was conveyed directly upon the GnRH neuroendocrine system.

Because of its key role in reproduction, there has been avid interest in identifying the location of kisspeptin neurons, and characterizing the neural circuitry by which kisspeptin acts to stimulate GnRH release and regulate reproductive neuroendocrine function (Oakley et al., 2009). An understanding of the functional role of kisspeptin signalling in the brain depends on the anatomical framework within which kisspeptin acts, i.e., knowing the location of neuronal cell bodies that synthesize the peptide, and their afferent/efferent connections. The primary aim of this review is to update our current understanding of the anatomical organization of the kisspeptin network; in this context, we would note that there has been one previous, excellent review of the neuroanatomy of the kisspeptin system (Mikkelsen and Simonneaux, 2009). However, in light of the recent addition of information from a wider variety of mammalian species, we viewed it as timely and worthwhile to re-evaluate the range of data reported to see where consistent patterns might emerge concerning the organization of the kisspeptin neural network. In addition, we review anatomical evidence of steroid receptor colocalization in kisspeptin neurons, findings supporting the existence of direct connections between kisspeptin and GnRH neurons, and recent evidence of phenotypic heterogeneity among subsets of kisspeptin cells which may contribute to their physiological functions. Finally, we end with a consideration of current gaps in this knowledge and some suggestions of future studies to fill those gaps.

2. Distribution of Kisspeptin Cells and Fibers in the Mammalian Brain

The location of kisspeptin cell bodies in the mammalian brain has been examined by two primary techniques: in situ hybridization (ISH) to detect cells expressing *Kiss1* mRNA transcripts, and immunocytochemistry (ICC), using either fluorescent or histochemical detection methods, to visualize kisspeptin peptide (Table 1). Initially, the use of ICC to detect kisspeptin-positive cell populations and fibers was confounded by the use of antibodies that cross-reacted with other members of the RFRP peptide family (Brailoiu et al., 2005). More recently, an antibody generated by Caraty and colleagues targeted against the C-terminal end of kisspeptin has been shown to be specific in a number of species both by careful preabsorption controls (Clarkson et al., 2009;Franceschini et al., 2006;Goodman et al., 2007) and the use of *Kiss1* knockout mice as negative controls (Clarkson et al., 2009). Studies using other kisspeptin antibodies have performed similar controls (Greives et al., 2007;Ohkura et al., 2009;Ramaswamy et al., 2008). Thus in our analysis of the location of kisspeptin cells and fibers (Tables 1 and 2) we have omitted ICC studies that utilized antibodies which have been shown to cross-react with other RFRP peptides (e.g., from Phoenix Pharmaceuticals) and where appropriate controls for such cross-reactivity are lacking.

The most consistent population of kisspeptin neurons identified across different mammalian species is the group located in the arcuate (ARC) nucleus (infundibular nucleus in humans). To date, this cell group has been identified, either by ISH or ICC, in humans, monkeys, sheep, rats, mice, hamsters, goats and horses (see Table 1 for references). In rodents, this population appears to be distributed throughout all rostral-caudal levels of the ARC (Gottsch et al., 2004;Smith et al., 2005), whereas in sheep, primates, goats and horses, they are located primarily at middle and caudal levels of the nucleus (Franceschini et al., 2006;Goodman et al., 2007;Ramaswamy et al., 2008).

In addition to the arcuate population, kisspeptin cell bodies have also been identified in the preoptic region by ISH or ICC in humans, monkeys, sheep, rats, mice and hamsters (Table 1). There are species differences in the precise location, and neurochemical phenotype, of kisspeptin cells in this region. In mice, rats and hamsters, preoptic kisspeptin cells are located in the anteroventral periventricular nucleus (AVPV) and extend as a continuum into the adjacent periventricular preoptic nucleus (PeN) (Gottsch et al., 2004;Smith et al., 2005;Smith et al., 2006); as discussed in more detail below, a subset of AVPV kisspeptin cells colocalize tyrosine hydroxylase (Kauffman et al., 2007b), galanin (Vida et al., 2009), which are present in other AVPV cells as well. In the sheep, monkey and human, kisspeptin cells are located at similar rostral-caudal levels in the preoptic area, although they are not directly adjacent to the third ventricle and appear to be more scattered than kisspeptin cells in the AVPV (Franceschini et al., 2006;Goodman et al., 2007;Hrabovszky et al., 2010;Smith et al., 2010). Furthermore, in the sheep, there appears to be no clear homolog to the AVPV, since the other neurochemical cell types that comprise this nucleus (e.g., dopamine, galanin (Herbison, 2008)) are not present as a well-defined cell group in the periventricular preoptic region (Lehman, unpublished observations). Whether the kisspeptin cells of the AVPV in rodents, and of the preoptic area (POA) in sheep and primates, are homologous to each other remains an open question and awaits use of additional markers. For the purpose of this review, we will refer to these subsets of neurons independently as the AVPV and POA populations, and consider both of them as cell groups in the 'preoptic region' (Table 1). It should be noted, however, there are a few species examined to date in which the presence of an AVPV, POA or other preoptic kisspeptin population has yet to be confirmed. For example, in goats, kisspeptin cells were not observed in the preoptic region despite the presence of large numbers of cells in the ARC in the same brains (Ohkura et al., 2009;Wakabayashi et al., 2010). However, these studies were performed using castrated male animals, and since kisspeptin expression in the AVPV and POA appears to be dependent on the presence of gonadal steroids (see below), these cells may not have expressed sufficient amounts of peptide to be detectable in castrated males. In the horse mare, one study (Magee et al., 2009) did identify POA neurons but used an antibody that has been questioned with regards to specificity (Goodman et al., 2007); another study in the female horse which used the more specific Caraty antibody (Decourt et al., 2008;Magee et al., 2009) failed to detect POA kisspeptin neurons. Hence the question of whether a kisspeptin cell population is present in the preoptic region of all mammals remains to be determined.

Total kisspeptin cell number appears to differ between the ARC and preoptic region populations, with greater numbers of cells seen in the ARC than POA in humans (Rometo et al., 2007) and sheep (Smith et al., 2007), in the ARC than AVPV in rats (Adachi et al., 2007; Kauffman et al., 2007b; Smith et al., 2006) based on ISH. It should be noted that the gonadal steroid, estradiol, has, in general, an opposite effect on each of these populations, stimulating *Kiss1* mRNA and peptide in the preoptic region[1] and inhibiting it in the ARC (Kauffman et al., 2007b; Smith et al., 2005; Smith et al., 2006; Smith et al., 2008). Thus differences between the number of preoptic region and ARC kisspeptin cells detected in these studies might simply be a reflection of the hormonal status of the animals used. However, even when the influence of steroidal milieu is taken into account (e.g., comparing kisspeptin cell number in the ARC of ovariectomized animals with preoptic region cell number in OVX animals treated with estradiol), the absolute number of detectable kisspeptin cells is higher in the arcuate nucleus than the preoptic region. For example, the POA of estradiol-treated OVX sheep during the breeding season contains approximately 100 *Kiss1*-

[1]An exception to this is in the monkey, where quantitative PCR showed no difference in *Kiss1* mRNA levels in the POA between ovariectomized and gonadally-intact female rhesus monkeys.

expressing cells (Smith et al., 2008); by contrast, the ARC of OVX sheep contains more than 400 cells (Smith et al., 2008). Similarly in the rat, the number of *Kiss1*-mRNA expressing neurons in the AVPV of estradiol-treated OVX females contains approximately 120 cells (Kauffman et al., 2007b) whereas the ARC of OVX females without steroid treatment contains approximately 200 *Kiss1* cells (Kauffman et al., 2007b). Thus, it appears that the ARC kisspeptin cell population contains consistently greater numbers of cells than the kisspeptin population in the preoptic region, even though the level of kisspeptin expression in these cells is influenced by gonadal hormones.

In addition to neuronal populations in the ARC and preoptic region, there are a few additional, smaller populations of kisspeptin cells that have been reported, and are variable among species. Perhaps the most controversial of these is a small group of scattered kisspeptin-immunoreactive neurons in the dorsomedial hypothalamus (DMH), that is seen in the brains of sheep (Franceschini et al., 2006), mice (Clarkson and Herbison, 2006; Clarkson et al., 2009) and horse mares (Decourt et al., 2008), but not in the rat (Desroziers et al., 2010; Kauffman et al., 2007b) or hamster (Greives et al., 2007). Although the genuine nature of kisspeptin localization in these cells was originally questioned because of their detection by non-specific antibodies that cross-reacted against RFRP3 cells in this region, as well as the failure to detect them by ISH, recent ICC studies have confirmed their presence in mice, sheep and horses, using the highly-specific Caraty antibody. The inability to detect these cells by ISH may be due to either their few number and scattered distribution, and/or to low levels of mRNA expression. In addition to kisspeptin cells in the DMH, in sheep and horses, a few kisspeptin-immunoreactive cells have also been reported in the ventromedial hypothalamic nucleus, however, in the sheep, these were detected using ISH and have not been confirmed with ICC using the Caraty antibody (Estrada, 2006), and in the horse (Magee et al., 2009), were detected with an antibody questioned for its specificity (Magee et al., 2009). In the monkey, a small number of kisspeptin cells extend from the ARC population directly into the median eminence (Ramaswamy et al., 2008), and in the human, kisspeptin neurons have been identified in the infundibular stalk (Hrabovszky et al., 2010). Finally, there is strong evidence from ISH studies in the mouse that a distinct population of kisspeptin cells exists outside the hypothalamus, in the medial amygdala (Gottsch et al., 2004; Kauffman, 2007) and BNST (Gottsch et al., 2004). Kisspeptin cells in the medial amygdala have not yet been identified in other species, however, a small number of *Kiss1* expressing cells in the BNST have been recently reported in the female rhesus monkey (Smith et al., 2010). The presence of kisspeptin-immunoreactive cell bodies in the medial amygdala or BNST have not yet been reported, although there are kisspeptin-positive fibers in both regions of the mouse (Clarkson and Herbison, 2006; Clarkson et al., 2009).

In addition to hormonal influences, there is evidence of clear sexual dimorphism in kisspeptin expression in all species examined to date, and gender is a factor that needs to be taken into account when evaluating the presence or absence of specific populations using either ISH or ICC. In rodents, the AVPV kisspeptin population is sexually differentiated, with females expressing a significantly greater number of *Kiss1*/kisspeptin-ir neurons than males (Ansel et al., 2010; Clarkson and Herbison, 2006; Smith et al., 2006). This dimorphism cannot be accounted for by differences in the adult hormonal milieu, because both intact and gonadectomized males and females show this sex difference, as well as gonadectomized males and females replaced with the same gonadal steroid (Adachi et al., 2007; Kauffman et al., 2007b). Thus differences in the AVPV are likely due to the organizational influence of gonadal steroids during development (Kauffman, 2009). In contrast to the AVPV population, kisspeptin cells in the ARC of rodents show no sex difference in their number. However, in sheep, both POA and ARC kisspeptin populations show sex differences, with greater numbers of cells in ewes than rams (Cheng et al., 2010). The sex difference in the ARC population also appears to be due to organizational effects of

gonadal steroids since ovariectomized pubertal ewes show greater numbers of cells than castrated rams (Nestor et al., 2010), but steroid replacement studies have yet to be done. In addition, sex differences in the same direction have recently been reported in the infundibular nucleus of humans with greater numbers of kisspeptin cells in females than males (Hrabovszky et al., 2010). In the human preoptic region, kisspeptin-immunoreactive neurons were consistently visualized in females, while none were seen in any of the male brains examined (Hrabovszky et al., 2010). Sex differences in kisspeptin cell number in other species, including monkeys, have not yet been reported, nor is it known whether the differences reported are due to gender-related cell death, as in the case of the sexual dimorphic nucleus of the preoptic area (Davis et al., 1996), or due to changes in gene/peptide expression. The reason for the difference in which kisspeptin populations are sexually differentiated between rodents, and sheep and humans, may lie in the functional roles that these areas play in the preovulatory GnRH surge, which is present in females, but not males. In rodents, the AVPV has been shown as a critical region driving the preovulatory GnRH surge, as lesions of this area prevent the estradiol-induced surge (Wiegand, 1980), whereas the ARC region has been implicated as essential for the steroid-induced preovulatory surge in sheep (Caraty et al., 1998) and primates (Hess et al., 1977; Krey et al., 1975). Thus, sexual differences in the ARC (infundibular) kisspeptin population in sheep and humans may reflect the importance of this cell group in the generation of the GnRH surge, compared to rodents in which the AVPV plays the predominant role.

Axonal fiber projections arising from kisspeptin cell populations have been analyzed by ICC in a range of species (Table 2). Kisspeptin fibers are reported consistently in the same regions where a majority of kisspeptin cells bodies are located, namely the ARC and preoptic region, with denser kisspeptin fibers reported in the ARC than in the preoptic region for all species. Besides the ARC, the densest accumulation of kisspeptin fibers is seen in the internal zone of the median eminence (Clarkson and Herbison, 2006;Decourt et al., 2008;Desroziers et al., 2010;Franceschini et al., 2006;Hrabovszky et al., 2010;Ramaswamy et al., 2010;Wakabayashi et al., 2010); In sheep (Franceschini et al., 2006), monkeys (Ramaswamy et al., 2008), rats (Desroziers et al., 2010) and goats (Wakabayashi et al., 2010), kisspeptin fibers have also been seen in the external zone of the median eminence where GnRH fibers terminate on portal vessels. However, it is noteworthy that in each of these species, axons and terminals in the external zone are much fewer in number and density than the kisspeptin fibers in the internal zone of the median eminence. A caveat is this observation may reflect more active release (and depletion) of peptide from fibers in the external than internal zone; comparison of kisspeptin fiber staining in the external zone under different endocrine conditions, presumably reflecting different patterns of endogenous kisspeptin release, might be useful in addressing this possibility.

Thus far, the mouse, rat and human, are the only species in which kisspeptin fibers have been thoroughly mapped outside of the ARC, POA and median eminence, and studies in each of these species have used the Caraty antibody. The overall comparison reveals many areas where kisspeptin fibers are found in common in mouse, rat, and human; these include the ARC, AVPV (including PeN), internal zone of the median eminence, PVN, DMH and lateral septum (Table 2). However, there are some differences: for example, in the mouse but in the rat, kisspeptin-positive fibers are seen in the paraventricular nucleus of the thalamus, medial amygdala, periaqueductal gray, and locus coeruleus (Clarkson et al., 2009;Desroziers et al., 2010). In the rat but not the mouse, kisspeptin fibers were reported in the suprachiasmatic nucleus and septohypothalamic area (Desroziers et al., 2010), while in humans, kisspeptin fibers are seen within the VMH (Hrabovszky et al., 2010) while in the rat (Desroziers et al., 2010) and mouse (Clarkson et al., 2009), fibers surround the VMH but do not enter it. Since these studies used antisera with the same specificity (Caraty anti-Kp-10 #564 and 566), and since the immunocytochemical protocols were largely the same, there

may be genuine species difference in the distribution of kisspeptin fibers in these regions. As discussed below, the discovery of a unique set of neuropeptide markers of the ARC kisspeptin populations (Goodman et al., 2007;Navarro et al., 2009;Wakabayashi et al., 2010) has made it possible to use multiple-label ICC to map out fiber projections specific to the ARC population, along with identification of its postsynaptic targets.

3. Steroid Receptor Colocalization in Kisspeptin Neurons

A substantial body of work has implicated kisspeptin neurons as primary mediators of gonadal steroid feedback control of GnRH release in mammals (Lehman et al., 2010; Roseweir and Millar, 2008; Smith, 2008). One of the major pieces of evidence for this role is the high degree of colocalization of kisspeptin cells with gonadal steroid receptors, specifically those for estradiol, progesterone and testosterone (Table 3). In general, studies using multiple-label ISH or ICC to evaluate colocalization have revealed fairly similar pictures of the extent of colocalization in different species. For example, in the ARC, studies in rats, mice and sheep reveal a similar high degree of colocalization of estrogen receptor-alpha (ER-α), progesterone receptor (PR), and androgen receptor (AR) in kisspeptin neurons, ranging from 70–99% (Table 3; Fig. 1, A–C). In the sheep POA, approximately 50% of the kisspeptin neurons coexpress ER-α (Franceschini et al., 2006), and in the rodent AVPV, a range from 62–99% in colocalization of ER-α and PR has been reported (Adachi et al., 2007; Clarkson et al., 2008; Smith et al., 2005; Smith et al., 2006). The difference between sheep and rodents may be due to the different techniques employed (ISH in most rodent studies vs. ICC in sheep), or may reflect species differences in the functional roles that these populations may serve (i.e., the preovulatory GnRH surge). Nonetheless, there is a consistent, high degree of colocalization of ER-α, PR and AR in kisspeptin cells across species, supporting this feature as a key characteristic of the kisspeptin neuronal network. By contrast, the percentage of kisspeptin cells in both the ARC and preoptic region that colocalize estrogen receptor-beta (ER-β) is much less, ranging from 11–25% in the ARC and 21–43% in the AVPV (Table 3). Thus, the effects of estradiol on both ARC and AVPV kisspeptin populations are likely mediated primarily by ER-α, consistent with evidence that this isoform mediates physiological control of GnRH secretion by estradiol feedback (Smith et al., 2005; Wintermantel et al., 2006). For the most part, other nuclear steroid receptors have not yet been studied for colocalization in kisspeptin cells. One exception is the type II glucocorticoid receptor which has been shown to be present in approximately 50% of dynorphin neurons in the ARC (Oakley, 2009); given near complete colocalization of dynorphin and kisspeptin in the ARC (see below), glucocorticoid receptors are almost certainly co-expressed in ARC kisspeptin cells as well.

4. Anatomical Sites of Interaction between Kisspeptin and GnRH neurons

Given the expression of the Kiss1 receptor (Kiss1R) within GnRH neurons (Han et al., 2005; Herbison et al., 2010; Irwig et al., 2004), as well as the demonstration of direct stimulatory effects of kisspeptin upon GnRH cell electrophysiology (Han et al., 2005; Pielecka-Fortuna et al., 2008; Roseweir et al., 2009), it has been presumed that kisspeptin neurons must synapse directly upon GnRH neurons. Nonetheless, while a number of studies have shown contacts between kisspeptin fibers and GnRH neurons at a light microscopic level, there is currently no direct electron microscopic (EM) evidence of kisspeptin terminals synapsing directly on GnRH somas or dendrites. Perhaps the best evidence shy of EM comes from dual-label studies using the confocal microscope where optical sections of 1 micron or less in thickness can be analyzed for close associations between kisspeptin and GnRH neurons. Thus far, confocal images of kisspeptin terminals in direct apposition to GnRH cell bodies have demonstrated in monkeys, sheep, mice and horses (Clarkson and Herbison, 2006; Decourt et al., 2008; Ramaswamy et al., 2008; Smith et al., 2008). As in the

case of other kisspeptin fibers, it should be noted that the detection of close contacts with GnRH neurons depends on the level of peptide present in those presynaptic terminals, and thus may vary according to gonadal hormone levels. Studies in monkeys (Ramaswamy et al., 2008; Smith et al., 2010), sheep (Smith et al., 2008), and mice (Clarkson and Herbison, 2006), have quantified the number of kisspeptin close contacts onto GnRH neurons located in either the POA and/or mediobasal hypothalamus (MBH). In the case of POA GnRH neurons, evidence from female mice (Clarkson and Herbison, 2006) and sheep (Smith et al., 2008) show that approximately 41–55% of GnRH cells receive at least one kisspeptin positive close contact; in the female sheep this percentage is much higher (95%) for GnRH cells located in the MBH. However, in female rhesus monkeys, the percentage of GnRH cells receiving input is much lower, with approximately 5–15% of POA GnRH neurons, and 20% of MBH GnRH neurons, receiving at least one kisspeptin-positive contact (Smith et al., 2010). In addition to species and regional differences, there is also evidence for sex differences in kisspeptin inputs onto GnRH neurons. Specifically, Clarkson and Herbison (2006) showed that a greater percentage of POA GnRH neurons in female brains (40%) receive direct kisspeptin contacts than in the male brain (10%) (Clarkson and Herbison, 2006). A sex difference in kisspeptin input to GnRH neurons may also be present in the monkey, where 33% of MBH GnRH neurons in the male receive one or more kisspeptin-positive inputs (Ramaswamy et al., 2008), as opposed to 20% of MBH GnRH neurons in the female (Smith et al., 2010). However, these observations are based on separate studies, and, as in the case of cell number comparisons, studies of sex difference in kisspeptin inputs need to be replicated with comparisons between gonadectomized animals, as well as gonadectomized animals with steroid replacement.

Confocal evidence of close contacts is not the same as direct EM level observations of synapses, but the additional detection of synaptic markers allows confocal multiple-label ICC to be used as a reliable proxy for the presence of synapses. For example, we have shown that direct contacts between synapsin-positive terminals and neurochemically-identified postsynaptic cells, seen under a light microscope in thin, 1 μm, sections, are always predictive of synapses when the same material is viewed at an EM level (Adams et al., 2006). Thus, analysis of 1 μm thick confocal sections that are triple-labeled for kisspeptin, synaptophysin (another synaptic marker), and GnRH, should provide strong evidence of synaptic inputs onto those cells. Using this approach, we have demonstrated that almost all kisspeptin close contacts on ovine GnRH neurons in the MBH are also synaptophysin-positive, and thus likely represent bona fide synaptic inputs (Smith et al., 2008; Fig. 1D). This same approach should be very useful for assessing the relative contribution of kisspeptin compared to other types of inputs (e.g., GABAergic) onto GnRH neurons, and the variation in that array of inputs with respect to sex, endocrine status, or age.

In addition to contact onto GnRH cell bodies, studies in the rhesus monkey and horse have noted the close associations of kisspeptin fibers with GnRH terminals in the external zone of the median eminence (Decourt et al., 2008; Ramaswamy et al., 2008). Anterograde tracing from neurokinin B cells in the ARC (that colocalize kisspeptin, see below) in rodents (Krajewski et al., 2010), as well as retrograde tracing studies from the median eminence in the sheep (Lehman et al., 2010), confirms this projection, and suggest that the ARC population is a major source of this kisspeptin input onto GnRH terminals. The ability of kisspeptin to affect the release of GnRH from murine hypothalamic slices lacking GnRH cell bodies has provided evidence for the median eminence as a potential site of action for kisspeptin in its control of GnRH secretion (d'Anglemont de Tassigny et al., 2008). Observations of close interactions between kisspeptin and GnRH fibers in the external zone provides an anatomical substrate for this site of action; however, as noted above, the number of kisspeptin-positive fibers in the external zone of the median eminence is sparse and variable among species, especially compared with the high density of these fibers in the

internal zone (Table 2). It may be that if kisspeptin acts upon GnRH terminals in this region it does so via paracrine signaling, with kisspeptin released in the internal zone diffusing to the external zone. The possibility of paracrine signaling within the median eminence has been noted in older work where tancytes and other glial cells elements have been postulated to mediate the transport of molecules across internal and external zones (Agnati et al., 1995; Lehman, 2000).

5. Heterogeneity among Kisspeptin Cell Populations

Recent evidence suggests that not all kisspeptin neurons are the same phenotypically, and, that some of these anatomical differences may underlie functional differences in the role of specific kisspeptin population in positive and negative steroid feedback controls of GnRH secretion (Dungan et al., 2006; Kauffman et al., 2007a). In particular, there is consistent evidence in the mouse, rat, sheep, goat, and human that kisspeptin cells in the ARC, but not in the POA/AVPV, colocalize two other neuropeptides shown to be important in the control of GnRH secretion, neurokinin B (NKB) (Topaloglu et al., 2009) and dynorphin (DYN) (Goodman et al., 2004) (Fig. 1E–F; Fig. 2). Nearly all ARC kisspeptin neurons in these species colocalize NKB and DYN (Goodman et al., 2007; Lehman et al., 2010) because of this, and for convenience, we have termed this cell population, the KNDy (Kisspeptin. Neurokinin B, Dynorphin) cells (Cheng et al., 2010). KNDy cells likely play multiple roles in control of GnRH secretion. Evidence from sheep and rodents suggest they are critical for conveying the negative feedback influence of estradiol and progesterone onto GnRH neurons (Goodman et al., 2004; Smith et al., 2007); in the sheep they may also play a role in the positive feedback influence of estradiol to induce the preovulatory GnRH surge (Lehman et al., 2010; Smith et al., 2009). Another interesting characteristic of KNDy cells in the ARC is that they possess extensive reciprocal connections with each other, confirmed at both light microscopic and EM levels, forming what appears to be an interconnected network (Burke, 2006; Foradori, 2006; Krajewski et al., 2010). Reciprocal connections appear much less abundant among POA kisspeptin neurons in sheep (Lehman et al., 2010), which, if homologous to preoptic kisspeptin cells in the rodent, correlates with the lack of effect of kisspeptin on the electrophysiological firing of AVPV kisspeptin neurons in the mouse (Ducret et al., 2010). Thus, the reciprocal coupling of the ARC kisspeptin population, together with the colocalization of NKB and dynorphin receptors, may be critical in enabling these neurons to fire synchronously with each other. Observations of rhythmic, multiunit activity recorded from the location of kisspeptin neurons in the ARC is consistent with this (Ohkura et al., 2009), and the temporal association of these electrophysiological rhythms with GnRH/LH pulses has led to speculation that they comprise a critical component of the “GnRH pulse generator” (Lehman et al., 2010; Navarro et al., 2009; Wakabayashi et al., 2010). Observations of changes in the shape of each GnRH pulse in response to an opioid antagonist (Goodman, 1995) and alterations in both multi-unit activity (Wakabayashi et al., 2010) and LH pulse frequency induced by treatment with opioid (dynorphin) and NK3R (NKB) antagonists (Goodman et al., 2010) are consistent with this hypothesis.

The presence of the unique set of KNDy neuropeptides for the ARC kisspeptin population has provided the opportunity to use multiple-label ICC to analyze the projections of this population, including its potential inputs to GnRH neurons. Thus far, studies in the rat have used a combination of dynorphin and NKB (Burke et al, 2006), as well as the combination of kisspeptin and NKB (Kirigiti et al., 2009), to trace projections from KNDy cells. Dual-labeled projections from KNDy neurons have been reported in each of the areas shown in Table 2 where single-labeled kisspeptin fibers are found, albeit in lesser numbers. In addition, dual-labelled KNDy fibers are found in the external zone of the median eminence (Burke, 2006), and dynorphin fibers can be seen in direct contact with GnRH terminals in

the median eminence at an electron microscopic level (Lehman et al., 2010). In addition, the colocalization of dynorphin and NKB in axon terminals has been used to show direct contacts of KNDy neurons onto GnRH cell bodies in the sheep (Lehman et al., 2010).

While kisspeptin cells in either the POA or the AVPV do not express either neurokinin B or dynorphin (Goodman et al., 2007; Navarro et al., 2009), there is evidence that a majority of AVPV kisspeptin population in the rodents colocalizes galanin, a neuropeptide implicated in female reproductive function (Vida et al., 2009), and that a subset also colocalizes tyrosine hydroxylase, a marker for dopaminergic neurons (Kauffman et al., 2007b) (Fig. 2). Subsets of AVPV neurons also express GABA and glutamate, and the extent to which either AVPV or POA kisspeptin neurons may colocalize these neurochemicals has not yet been examined. However, neurotensin, which is also expressed by AVPV neurons, is not colocalized with kisspeptin (Dungan Lemko et al., 2010). Kisspeptin cells of the amygdala (Kauffman, 2007) have not yet been examined for the co-expression of other neuropeptides/transmitters, although based on studies of the phenotype of cells in the medial amygdala that project to the preoptic area and GnRH cells, it seems likely that some of these may colocalize the neuropeptides, cholecystokinin and/or substance P (Simerly, 1989).

6. Summary and Future Directions

Key features of the kisspeptin neural network and its interactions with GnRH neurons, based on our current knowledge, are summarized in Fig. 2. Kisspeptin cell are found consistently in two major cell populations, one located in the ARC and the other in preoptic region, in either the AVPV or POA. While the ARC population is highly conserved among species, there is variation in the location and phenotype of preoptic kisspeptin neurons. In rodents, kisspeptin cells comprise a component of the AVPV, but in sheep, monkeys and humans, they appear to be more scattered and an AVPV homologue is not evident. In may be, in fact, that the connections and neurochemical features of the AVPV and its kisspeptin cells are critical for the functional role of this population in rodents, and may underlie differences between rodents and other species in the control the GnRH surge (Caraty et al., 1998;Krey et al., 1975;Wiegand, 1980). In addition to the ARC and preoptic populations, is also evidence for smaller populations of kisspeptin neurons in the DMH, BNST and medial amygdala, but it is not clear whether they are consistently seen across species, nor has their functional role(s) been identified. Kisspeptin cells of the ARC and preoptic populations differ in their neurochemical phenotype: virtually all ARC kisspeptin cells contain both neurokinin B and dynorphin, while subsets of AVPV kisspeptin cells express galanin and/or tyrosine hydroxylase.

There are several sites of demonstrated and potential direct interactions between the kisspeptin and GnRH neuronal networks (Fig. 2, numbers 1–2). Evidence from confocal, multiple-label studies using synaptic markers strongly supports the existence of direct synaptic connections onto GnRH cell bodies (Smith et al., 2008), some of which arise from the ARC kisspeptin population.. Transneuronal tracing studies (Wintermantel et al., 2006) and studies using conventional tracers (Gu and Simerly, 1997;Hahn and Coen, 2006;Polston and Simerly, 2006) have demonstrated that cells of the AVPV provide direct input to GnRH neurons; unpublished data (Herbison, 2008) suggests that these inputs arise at least in part from AVPV kisspeptin cells, although there are likely also inputs from other neurochemical subsets of the AVPV (glutamate, GABA). In addition, to direct inputs from kisspeptin cells onto GnRH cell bodies, there is also evidence for potential inputs at the level of GnRH terminals in the median eminence. EM observations have confirmed that dynorphin terminals, presumably arising from the ARC, establish direct contacts with GnRH terminals (Lehman et al., 2010). Nonetheless, evidence is still needed at an EM level to confirm that kisspeptin-positive terminals are in direct contact with GnRH terminals in the median

eminence, as well as demonstration that Kiss1 receptors are present on the plasma membranes of those GnRH terminals.

A number of anatomical features support the view that kisspeptin cells form a reciprocally-innervated functional network, both within a given region and between different populations, and extending to include GnRH neurons. First, as noted above, kisspeptin cells of the ARC have extensive reciprocal connections with each other (Fig. 2, number 3) (Burke, 2006;Foradori, 2006;Goodman et al., 2007;Krajewski et al., 2010). Second, there is evidence that ARC kisspeptin cells (based on dual-labeling for kisspeptin and dynorphin) send direct projections to kisspeptin neurons in the sheep POA (Lehman, unpublished). Further, recent evidence suggests that approximately 40% of AVPV kisspeptin neurons in the mouse, in turn, project to the ARC (Yeo and Herbison, 2009), raising the possibility of reciprocal communication between these two populations (Fig. 2, number 4). Finally, in the monkey (Ramaswamy et al., 2008) and sheep (Lehman, unpublished), GnRH fibers have been shown to contact ARC kisspeptin neurons (Fig. 2, number 5), providing a route for two-way communication between GnRH and kisspeptin populations. Given that GnRH neurons are interconnected morphologically, at the level of their dendrites as well as axon terminals (Campbell et al., 2009), there is therefore potential for kisspeptin input to GnRH neurons, either at the level of their cell bodies or terminals, to influence the coordinated release of GnRH from many distributed neurons.

Many unanswered questions remain concerning the anatomical organization of this network. First, while some information has been obtained about the efferent connections of ARC and preoptic kisspeptin populations, projections from kisspeptin cells in the DMH, BNST and medial amygdala have not yet been examined, including the possibility that one or more of these populations also provides input to GnRH neurons. There is evidence from tract tracing studies that all three areas project to the preoptic region (Hahn and Coen, 2006; Simerly, 1986; Tillet et al., 1993) and specifically to GnRH neurons (Boehm et al., 2005; Pompolo et al., 2005; Yoon et al., 2005). Second, the sources of afferent inputs to each kisspeptin population need to be identified. One likely source of afferents to the AVPV kisspeptin population is the suprachiasmatic nucleus (SCN), given evidence that these kisspeptin neurons are involved in circadian regulation of the GnRH surge in rodents (Robertson et al., 2009) (Fig. 2). In fact, vasopressin and vasoactive intestinal peptide-expressing terminals originating from the SCN make contacts onto AVPV kisspeptin neurons, as shown recently by anterograde tract tracing combined with ICC (Vida et al., 2010). In the future, transgenic neuron-specific tracing, both anterograde and retrograde (transneuronal), could be used for defining the inputs to each kisspeptin population as it has for leptin-responsive cell populations (Leshan et al., 2010) and GnRH neurons (Boehm et al., 2005; Wintermantel et al., 2006; Yoon et al., 2005), respectively. Third, the identity of other postsynaptic targets of kisspeptin cells, besides GnRH, needs to be examined; for example, recent evidence suggests that kisspeptin plays a role in the regulation of prolactin via direct contacts of kisspeptin fibers onto A12 dopamine cells (Szawka et al., 2010). Finally, it will be important to know which cellular targets of kisspeptin cells actually express Kiss1R. While there is a considerable overlap between regions that express Kiss1R and kisspeptin fiber-immunoreactivity (Herbison et al., 2010), there are some examples of apparent receptor-ligand mismatch. For example, the dentate gyrus of the hippocampus is a region which contains a large number of Kiss1R-expressing cells (Herbison et al., 2010), although kisspeptin fibers have not been identified in that region. Conversely, some areas that have contain dense kisspeptin fibers, such as the ARC, are devoid of Kiss1R (Herbison et al., 2010). Whether these examples of receptor-ligand mismatch reflects the presence of other as yet unidentified receptors or ligands, or the influence of kisspeptin via extra-synaptic communication routes, remains to be seen.

In summary, converging data from a range of species suggests that the overall organization of the kisspeptin neuronal system in mammals is fairly consistent, and that direct anatomical projections to GnRH neurons, at the level of both cell bodies and terminals, are a common feature. In addition, there is growing recognition that kisspeptin is present as only one of several important peptides/neurotransmitters in this circuitry, and that the neural projections of the kisspeptin network are likely to include other neuroendocrine systems, as well as extend outside the preoptic-hypothalamic continuum. Indeed, the neurochemical and anatomical heterogeneity of the kisspeptin network is likely to be critical in defining the individual functional roles of subsets of kisspeptin neurons, and much important work remains to be done in order to define the structural framework for kisspeptin action in the brain.

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Abbreviations

AR	androgen receptor
ARC	arcuate nucleus
AVPV	anteroventral periventricular nucleus
BNST	bed nucleus of the stria terminalis
DMH	dorsomedial hypothalamus
DYN	dynorphin
EM	electron microscopy
ER-α	estradiol receptor-alpha
ER-β	estradiol receptor-beta
ICC	immunocytochemistry
ISH	*in situ* hybridization
LH	luteinizing hormone
LHA	lateral hypothalamic area
MBH	mediobasal hypothalamus
ME	median eminence
NKB	neurokinin B
OVX	ovariectomized
PeN	periventricular nucleus
POA	preoptic area
PR	progesterone receptor
PVN	paraventricular nucleus
RFRP	RFamide-related peptides
SCN	suprachiasmatic nucleus
SON	supraoptic nucleus

VMH ventromedial hypothalamus

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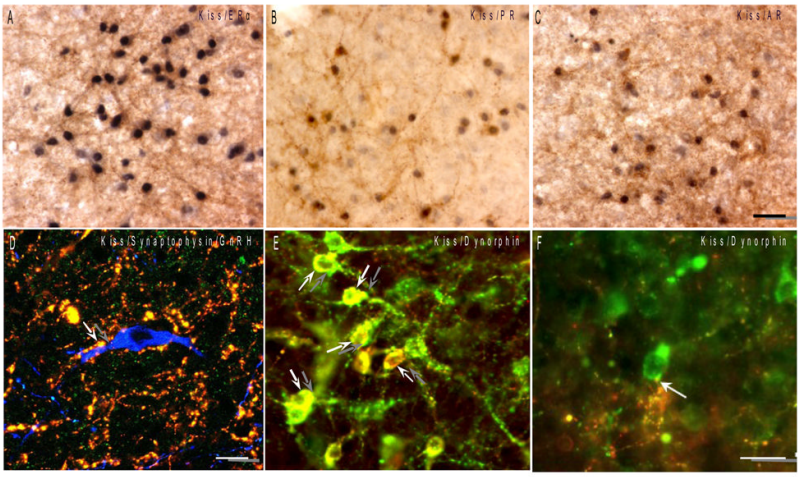

Fig. 1.

A–C: Colocalization of gonadal steroid receptors in kisspeptin neurons. Dual immunostained sections of sheep ARC showing high degree of colocalization of nuclear ER-α, PR and AR (blue-black) in kisspeptin cells (brown). Bar = 50 μm. **D: Kisspeptin synaptic contacts onto a GnRH neuron.** Confocal optical section (1 μm thick) of a triple-labeled section showing a terminal labelled with both kisspeptin (red) and synaptophysin (green) in direct contact with an ovine GnRH (blue) cell body (modified from Smith et al., 2008). Bar = 10 μm. **E–F: Phenotypic heterogeneity between ARC and preoptic kisspeptin neurons.** E: Colocalization of the endogenous opoid peptide, dynorphin (red), in kisspeptin neurons (arrows; green) of the sheep ARC. F: By contrast, kisspeptin neurons (green) in the sheep POA do not colocalize dynorphin even though they receive input from dynorphin-positive fibers (arrow; red). Bar = 20 μm. (modified from Goodman et al., 2007)

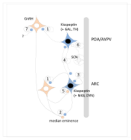

Fig. 2. Schematic horizontal section (top=rostral, bottom=caudal) showing the POA/AVPV and ARC kisspeptin populations in the mammalian hypothalamus, and their potential sites of interactions with GnRH neurons

Virtually all ARC kisspeptin neurons co-express neurokinin B and dynorphin (Goodman et al., 2007; Navarro et al., 2009; Wakabayashi et al., 2010), while subsets of AVPV kisspeptin neurons in the preoptic region express either galanin (Vida et al., 2009) or tyrosine hydroxylase (Kauffman et al., 2007b). Connections (solid lines, published data; dotted lines, unpublished data) and sites of interactions between kisspeptin and GnRH systems include: **1**) Direct projections from ARC and AVPV kisspeptin cells onto GnRH cell bodies (Clarkson and Herbison, 2006; Kinoshita et al., 2005; Krajewski et al., 2005; Lehman et al., 2010; Ramaswamy et al., 2008; Smith et al., 2008); **2**) Inputs from ARC kisspeptin cells onto GnRH terminals in the median eminence (Burke, 2006; Krajewski et al., 2005; Lehman et al., 2010; Ramaswamy et al., 2008); **3**) reciprocal connections among ARC kisspeptin cells that could be from the same or adjacent neurons (Burke, 2006; Foradori et al., 2002; Krajewski et al., 2010; Wakabayashi et al., 2010); **4**) Projections from ARC kisspeptin neurons to POA kisspeptin cells in the sheep (Lehman, unpublished), and from AVPV kisspeptin neurons back to the ARC (Yeo and Herbison, 2009); and **5**) projections from GnRH neurons back onto ARC kisspeptin cells (Ramaswamy et al., 2008). In addition, afferents to POA/AVPV kisspeptin cells from the suprachiasmatic nucleus (SCN) are indicated (**6**) (Vida et al., 2010), as well as the possibility that kisspeptin inputs to GnRH neurons in the POA or MBH may arise from other populations such as the DMH, BNST or medial amygdala (**7**).

Table 1

Distribution of Kisspeptin/*Kiss1* Cells in the Mammalian Nervous System

Species	*Preoptic Region*				*Medial*				ISH Reference	ICC Reference
	ARC	*POA*	*AVPV*[a]	*ME*	*DMH*	*VMH*	*Amygdala*	*BNST*		
Human	+++	+							(Rometo et al., 2007)	(Hrabovszky et al., 2010)
Monkey	+++	++		+				+	(Rometo et al., 2007; Shahab et al., 2005; Smith et al., 2010)	(Ramaswamy et al., 2008; Ramaswamy et al., 2010)
Sheep	+++	++			+	+			(Estrada, 2006; Smith et al., 2007; Smith et al., 2008; Smith et al., 2009)	(Cheng et al., 2010; Franceschini et al., 2006; Goodman et al., 2007; Smith et al., 2008; Smith et al., 2009)
Rat	+++		++						(Adachi et al., 2007; Irwig et al., 2004; Kauffman et al., 2007; Smith et al., 2006; Takase, 2009)	(Desroziers et al., 2010; Kinoshita et al., 2005; Takase, 2009)
Mouse	++		++		+		+	+	(Gottsch et al., 2004; Han et al., 2005; Kauffman, 2007; Smith et al., 2005a; Smith et al., 2005b)	(Clarkson and Herbison, 2006; Clarkson et al., 2009)
Hamster	++		++						(Ansel et al., 2010; Revel et al., 2006)	(Greives et al., 2007; Mason et al., 2007)
Goat	+++									(Ohkura et al., 2009; Wakabayashi et al., 2010)
Horse	+++				+					(Decourt et al., 2008; Magee et al., 2009)

+++, large (50–150); ++, moderate (15–50); +, few (<15 or numbers not reported);

[a]Includes cells in PeN;

[b]Caraty anti-Kp10 used for ICC detection

Table 2

Distribution of Kisspeptin fibers in the Mammalian Nervous System

Species	*ARC*	*POA*	*AVPV*[a]	*Internal ME*	*External ME*	*PVN*	*DMH*	*SON*	*BNST*	*Lateral Septum*	*Other Regions*	**Reference**
Human	++	++	++	++	+	+	+			+	+[b]	(Hrabovszky et al., 2010)
Monkey	++	+		++	+							(Ramaswamy et al., 2008; Ramaswamy et al., 2010)
Sheep	++	+		++	+		+					(Franceschini et al., 2006; Goodman et al., 2007; Smith et al., 2008)
Rat	++	+	+	++	+	++	+	+	+	+	+[c]	(Adachi et al., 2007; Desroziers et al., 2010; Kinoshita et al., 2005; Takase, 2009)
Mouse	++		+	++		+	+	+	+	+	+[d]	(Clarkson and Herbison, 2006; Clarkson et al., 2009)
Hamster	++		+	++								(Mason et al., 2007)
Goat	++	+		++	+							(Ohkura et al., 2009; Wakabayashi et al., 2010)
Horse	++	+		++								(Decourt et al., 2008)

++, dense fibers; +, moderate or few fibers; PVN, paraventricular nucleus

[a] Includes fibers in the median preoptic nucleus, AVPV, and PeN;

[b] Other regions include: VMH, LHA;

[c] Other regions include: suprachiasmatic nucleus, septohypothalamic area, medial septum;

[d] Other regions include: medial septum, paraventricular nucleus of the thalamus, medial amygdala, periaqueductal gray, locus coeruleus.

Table 3

Percentage of Kisspeptin/*Kiss1* Cells Colocalizing Gonadal Steroid Receptors in Female Mammals

Species	ARC				POA			AVPV		
	ER-α	ER-β	PR	AR	ER-α	ER-β	PR	ER-α	ER-β	PR
Sheep	93[a]		86[b], >95[c]	>85[d]	50[a]					
Rat	92[e], 70[f]	11[f]						90[e], 62[f]	21[f]	
Mouse	>99[g], 88[h]	25[g]						99[g], 65[i]	31[g]	67[i]

[a] Franceschini et al., 2006, ICC;

[b] Smith et al., 2007, ISH;

[c] Cheng et al., 2010, ICC, data from males and females;

[d] Lehman, unpublished, ICC;

[e] Adachi et al., 2007, ISH;

[f] Smith et al., 2006, ISH;

[g] Smith et al., 2005a, ISH;

[h] Smith et al., 2005b, ISH;

[i] Clarkson et al., 2008, ICC