Kisspeptin Signaling in the Brain

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Kisspeptin (a product of the *Kiss1* gene) and its receptor (GPR54 or Kiss1r) have emerged as key players in the regulation of reproduction. Mutations in humans or genetically targeted deletions in mice of either *Kiss1* or *Kiss1r* cause profound hypogonadotropic hypogonadism. Neurons that express *Kiss1*/kisspeptin are found in discrete nuclei in the hypothalamus, as well as other brain regions in many vertebrates, and their distribution, regulation, and function varies widely across species. Kisspeptin neurons directly innervate and stimulate GnRH neurons, which are the final common pathway through which the brain regulates reproduction. Kisspeptin neurons are sexually differentiated with respect to cell number and transcriptional activity in certain brain nuclei, and some kisspeptin neurons express other cotransmitters, including dynorphin and neurokinin B (whose physiological significance is unknown). Kisspeptin neurons express the estrogen receptor and the androgen receptor, and these cells are direct targets for the action of gonadal steroids in both male and female animals. Kisspeptin signaling in the brain has been implicated in mediating the negative feedback action of sex steroids on gonadotropin secretion, generating the preovulatory GnRH/LH surge, triggering and guiding the tempo of sexual maturation at puberty, controlling seasonal reproduction, and restraining reproductive activity during lactation. Kisspeptin signaling may also serve diverse functions outside of the classical realm of reproductive neuroendocrinology, including the regulation of metastasis in certain cancers, vascular dynamics, placental physiology, and perhaps even higher-order brain function. **(*Endocrine Reviews* 30: 713–743, 2009)**

"*Somewhere, something incredible is waiting to be known.*"

—Carl Sagan (1934–1996), astronomer, cosmologist, writer

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Abbreviations: AR, Androgen receptor; ARC, arcuate nucleus; ARKO, aromatase knockout; AVPV, anteroventral periventricular nucleus; DAG, diacylglycerol; DBB, diagonal band of Broca; DMH, dorsomedial hypothalamus; ERα, α-estrogen receptor; ERE, estrogen response element; GPCR, G protein-coupled receptor; GPR54, G protein-coupled membrane receptor 54; IP_3, inositol triphosphate; -ir, immunoreactive; MBH, medial basal hypothalamus; ME, median eminence; NKB, neurokinin B; NVT, nucleus ventral tuberis; PeN, periventricular nucleus; PKC, protein kinase C; PLC, phospholipase C; POA, preoptic area; PR, progesterone receptor; RF-amide, Arg-Phe-NH_2; RFRP, RMRFamide-related peptide; RMRFamide, Phe-Met-Arg-Phe-NH_2; SCN, suprachiasmatic nucleus; TRPC, transient receptor potential canonical; VH, ventral hypothalamus; VMH, ventromedial hypothalamic nucleus.

I. Introduction

Since kisspeptin burst onto the scientific stage, it has soared to prominence—particularly with respect to its role in the neuroendocrine regulation of reproduction. Originally discovered as a metastasis-suppressor gene in 1996 (1), KISS1 was named for its role as a suppressor sequence (ss); the letters "KI" were appended to the prefix "SS" to form "KISS" in homage to the location of its discovery, Hershey, Pennsylvania, home of the famous "Hershey Chocolate Kiss." Although the term metastin had been coined for the 54-amino acid product of the *Kiss1* gene, another research group named the family of neuropeptides coded by the *Kiss1* gene, kisspeptins (2). Use of both terms continues to this day, with cancer biologists largely preferring the term metastin, whereas investigators in other fields have favored the term kisspeptin. In 2001, four independent groups identified kisspeptin (Table 1) as a high-affinity RFamide (Arg-Phe-NH_2) peptide ligand for a then orphan G protein-coupled membrane receptor, GPR54 (2–5). GPR54, now termed "Kiss1r" for its role as a kisspeptin receptor (Table 1), was initially described in the rat in 1999 (6), and shortly thereafter, the human homolog of GPR54 (KISS1R; then referred to as AXOR12 or hOT7T175) was identified (2–4). In 2003, kisspeptin-KISS1R signaling piqued the interest of reproductive physiologists when two independent research groups nearly simultaneously reported that mutations in *KISS1R* were associated with the idiopathic hypothalamic hypogonadism and impaired pubertal maturation found in their patients (7, 8). Moreover, studies of mice bearing targeted deletions of *Kiss1r* produced the same phenotypic anomaly of reproductive dysfunction (8, 9). Thus emerged the idea that kisspeptin-KISS1R signaling plays a vital role in reproduction. This review will focus on kisspeptin signaling in the brain—particularly as it relates to reproduction. Although a search for understanding the function of kisspeptin in cancer biology and organs outside of the brain remains a vigorous area of exploration, a detailed review of this subject is beyond the scope of this review and is summarized elsewhere (10).

II. Nomenclature

A recent review by Gottsch *et al.* (11) highlights the need for consistency in kisspeptin nomenclature and offers recommendations to unify terms across (and within) the various research fields. The kisspeptin receptor has been formerly referred to as AXOR12, hOT7T75, GPR54, KISS1R, KiSS1, and the metastin receptor (1–4, 12) (Table 1). There is more difficulty distinguishing among the kisspeptin gene, the mRNA, and the protein of different species. Gottsch *et al.* (11) suggest using the term kisspeptin in reference to the

TABLE 1. A summary of terms used to describe *KISS1/KISS1R* and kisspeptins and suggested usage [Modified with permission from Gottsch, *et al.* (11)]

	Current usage		Suggested usage	
	Gene/mRNA	**Peptide**	**Gene/mRNA**	**Peptide**
KISS1				
Rodent and other nonhuman species	KiSS-1, KiSS1, Kiss-1, Kiss1 (typically italicized for the gene and not for mRNA)	Metastin, Kisspeptin-145, -54, -14, -13, -10 (abbreviated Kp-145–Kp-10)	For rodents and all other nonhuman species: *Kiss1*	Kisspeptin-145–Kisspeptin-10, abbreviated Kp-145–10
	MGI format: *Kiss1*	Kisspeptin-1 (68-121) (aka metastin) or kisspeptin/ metastin (112-121)	*Kiss1* (mRNA)	With Kp-145 representing the entire 145 aa peptide, Kp-54 (aa 68-121), Kp-14 (aa 108-121), Kp-13 (aa 109-121), Kp-10 (aa 112-121)
		KiSS-1 peptide KiSS-1 protein		
Human	KiSS-1, KiSS1 (typically italicized for the gene and not for mRNA)	Metastin, Kisspeptin-145, -54, -14, -13, -10 (abbreviated Kp-145–Kp-10, KiSS-1)	KISS1	Kisspeptin (Kp)–145-10
	MGI format: *KISS1*	Human metastin 45-54	*KISS1* (mRNA)	Kp54 (metastin)
KISS1R				
Rodent and other nonhuman species	GPR54, *Gpr54* MGI format: *Kiss1r*	GPR54, Kiss1R	For rodents and all other nonhuman species: Kiss1r, *Kiss1r* (mRNA)	Kisspeptin (or Kiss1) receptor, abbreviated Kiss1r
Human	AXOR12, HOT7T175, *GPR54*, *KiSS1R*, metastin receptor	GPR54, KiSS1 (GPR54)	KISS1R	Kisspeptin (or KISS1) receptor, abbreviated KISS1R
	MGI format: *KISS1R*		*KISS1R* (mRNA)	

protein product(s) of the coding gene(s). Based on recommendations from the international committees established to standardize nomenclature (http://www.informatics.jax.org/mgihome/nomen/gene.shtml), *KISS1* and *Kiss1* should be used to represent the human and nonhuman kisspeptin genes, respectively (Table 1). The nonitalicized versions of the gene nomenclature should be used to refer to the protein products of *KISS1* (*i.e.*, KISS1 for human and Kiss1 for other species), although spelling-out "kisspeptin" is also appropriate (11). For the receptor, the Human Genome Nomenclature Committee recommends the use of *KISS1R* for the human kisspeptin receptor gene. Following the same convention that applies to the ligand, *Kiss1r* denotes the nonhuman receptor gene or mRNA, and KISS1R and Kiss1r denote the receptor protein for human and nonhuman species, respectively (11) (Table 1). The nomenclature referenced in Table 1 will be used throughout this review.

III. Biochemistry

A. Signaling

G protein-coupled receptors (GPCRs) transduce a variety of inputs to activate signaling pathways involved in diverse functions such as cell growth, proliferation, and migration. The GPCR superfamily can be classified into three subdivisions, *e.g.*, rhodopsin-, secretin-, and metabotropic glutamate receptor-like families (13). Typical of the rhodopsin family of GPCRs, Kiss1r contains seven transmembrane domains, with three glycosylation sites at the N terminus (5). Kiss1r is most similar to the galanin receptor family (~45% homologous), although it does not bind either galanin or galanin-like peptide (6). Screens for agonists that bind Kiss1r identified several neuropeptides of the RFamide and RWamide family (5). The RMRFamide (Phe-Met-Arg-Phe-NH_2)-related peptides (RFRPs), of which Kiss1 is a member, constitute a superfamily of neuropeptides that terminate with the sequence Arg-Phe-NH_2 and exist in all phyla (14, 15).

The binding of Kiss1r by Kiss1 peptide leads to the activation of G protein-activated phospholipase C (PLCβ), suggesting a $G\alpha_{q/11}$-mediated signaling pathway (2, 3, 16–18) (Fig. 1). PLCβ activation leads to the generation of the intracellular second messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG); these signaling molecules in turn mediate intracellular Ca^{2+} release and activation of protein kinase C, respectively (16, 18). Kisspeptin is thought to stimulate GnRH secretion by activating transient receptor potential canonical (TRPC)-like channels and inhibiting inwardly rectifying potassium channels (19), likely mediated by DAG and/or Ca^{2+}. Additionally, Kiss1r has been shown to stimulate arachidonic acid release and ERK1/2 and p38 activation, as well as Rho activation, which causes stress fiber formation (2, 20). Endogenous kisspeptin may activate Kiss1r via a ligand transportation pathway, in which initial binding of a ligand to the membrane is followed by lateral diffusion to the receptor (21). Current efforts are aimed at understanding more about the coupling of Kiss1r and G proteins. By examining the efficacy of signaling in various models of Kiss1r mutations, one recent report has identified the IL2-10 residue as a key player in the structural rearrangement of Kiss1r upon binding of the ligand, kisspeptin (22). This approach may shed new light on fundamental concepts regarding GPCR/G protein signaling of kisspeptin and other ligand-receptor interactions.

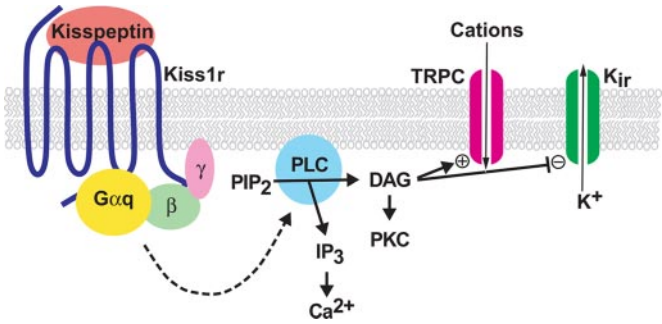

FIG. 1. Proposed mechanism of neuronal depolarization by kisspeptin binding to its receptor, Kiss1r. Kisspeptin binding to its GPCR, Kiss1r, activates the G protein, $G\alpha_q$, and PLC to cleave phosphatidylinositol 4,5-bisphosphate (PIP_2) into IP_3 and DAG. DAG activates a signal cascade by activating PKC, whereas IP_3 mobilizes calcium ions (Ca^{2+}), which participate in the cascade by activating other proteins. Membrane depolarization is caused by activation (+) of nonselective TRPC cation channels and inhibition (−) of inwardly rectifying potassium channels (K_{ir}), possibly through involvement of DAG.

Mutations and targeted deletions of *KISS1R/Kiss1r* cause profound hypogonadotropic hypogonadism in humans and mice. Various disabling mutations in *KISS1R* have been shown to occur in humans (23), and these mutations have involved deletion of as many as 155 nucleotides or as few as a single-nucleotide variant (L148S) in the second intracellular loop of the *KISS1R* gene (7, 24, 25). Recently, an activating mutation has also been described for the *KISS1R* in the human, which leads to precocious puberty (26). This mutation involves the substitution of proline for arginine at codon 386 (Arg386Pro), which causes prolonged intracellular KISS1R signaling in response to kisspeptin (26).

B. Active form

The initial product of the *Kiss1* gene is a 145-amino acid peptide, from which is cleaved a 54-amino acid protein known as kisspeptin-54 (27) (Fig. 2). In the full-length protein, the sequence of kisspeptin-54 is surrounded by pairs of basic residues, where furin or prohormone con-

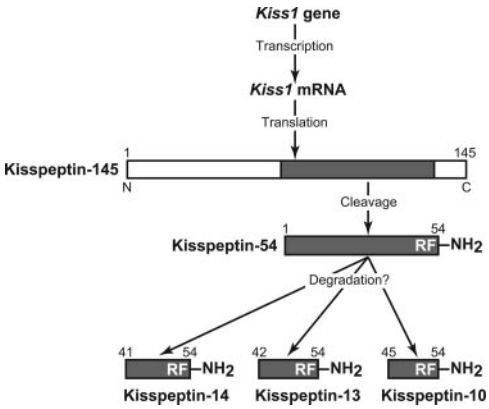

FIG. 2. Products of the *Kiss1* gene. *Kiss1* mRNA is transcribed from the *Kiss1* gene and translated to form a 145-amino-acid propeptide called kisspeptin-145. Shown are cleavage sites on the propeptide that lead to the production of the RF-amidated kisspeptin-54. Shorter peptides (such as kisspeptin-10, -13, and -14) share a common C terminus and RF-amidated motif with kisspeptin-54. Because no putative cleavage sites have been identified on the propeptide that would lead to synthesis of the shorter peptides, such peptides may be degradation products of kisspeptin-54. [Adapted with permission from Popa *et al.*, 2008 (202) © Annual Reviews].

vertases are thought to proteolytically cleave (2). There are also shorter peptides (kisspeptin-10, -13, and -14) that share a common RF-amidated motif with kisspeptin-54; collectively, they are termed kisspeptins. Although no obvious cleavage sites have been identified that would result in these shorter peptides, it has been suggested that kisspeptin-54 is unstable and may be proteolytically cleaved into the shorter products (2). All four peptides (kisspeptin-10, -13, -14, and -54) exhibit the same affinity and efficacy for the Kiss1r, indicating that the C-terminal end of the peptide is responsible for the binding and activation of the receptor, Kiss1r (2). Although all four kisspeptin products are biologically active (3), the *in vivo* relevance of the shorter peptides is as yet unknown.

Because members of the RFamide family of peptides often share several identical C-terminal amino acid residues, the generation of specific antisera has been a technical challenge. For example, many of the available KISS1 antisera raised against the shortened C-terminal human peptide cross-react with other members of the RFRPs (*i.e.*, RFRP-1 and RFRP-3) [preliminary data, Ref. 28]. RFRP-1 and RFRP-3 have been shown to stain strongly in the dorsomedial hypothalamus (DMH; an area that is not known to express *Kiss1* mRNA) with marked fiber projections in the arcuate nucleus (ARC) (29–34), which raises the possibility that previous kisspeptin antibodies demonstrating similar staining patterns may exhibit cross-reactivity with these related RFRPs. The reader is therefore advised to view kisspeptin antibody staining results with caution, unless unequivocal evidence for antibody specificity is established.

C. Analogs

Despite a critical role for kisspeptin signaling in both cancer and reproductive biology, only recently has progress been made in the development of novel ligands or pharmacologically therapeutic agents (agonists or antagonists). Instead of employing a random high-throughput screening approach for potential ligands, Orsini *et al.* (35) have identified a model kisspeptin pharmacophore utilizing a structure-activity relationship approach combining nuclear magnetic resonance, receptor binding, and functional assays. A structure-derived pharmacophore search has the advantage of yielding potential ligands that interact with the receptor in a binding mode similar to endogenous kisspeptin. The authors demonstrated that the kisspeptin-13 peptide has a relatively stable helix conformation from residues 7 to 13, with three functionally key residues (Phe9, Arg12, and Phe13) that lie on one face of the helix and define its pharmacophore site (35). Through amino acid substitution, Gutiérrez-Pascual *et al.* (36) have identified alanine at positions 6 and 10 as critical for kisspeptin-10 action at Kiss1r, pointing to potential modifications that could lead to new kisspeptin analogs. The stereochemistry of kisspeptin analog amino acids also appears to be of major importance; substitution of key residues with the d-isomer significantly decreases peptide agonist activity (37). Utilizing a structure-activity relationship approach, Tomita *et al.* (38–41) have identified several pentapeptide kisspeptin analogs as novel Kiss1r agonists. Molecules identified that mimic the key features of the pharmacophore site can act as full agonists, although with reduced potency compared with kisspeptin itself (35).

Several approaches have been used to block kisspeptin-Kiss1R signaling. Kinoshita *et al.* developed a monoclonal anti-rat kisspeptin antibody that, when infused in the preoptic area (POA), completely blocks the proestrus LH surge and inhibits estrous cyclicity (42) (Fig. 3). Most recently, Roseweir *et al.* (43) have developed several kisspeptin antagonists via amino acid substitution of kisspeptin-10 analogs. Based on its structure-activity profile, one potent and specific antagonist ("peptide 234") was selected for use in *ex vivo* and *in vivo* studies. This antagonist inhibits the kisspeptin-induced rise in LH secretion in mice and rats and blocks the postcastration LH rise in rodents and sheep, suggesting a powerful role of kisspeptin neurons in mediating the negative feedback action of sex steroids on the hypothalamic-pituitary gonadal axis (43). Furthermore, the antagonist inhibits kisspeptin-10-induced GnRH neuronal firing in the mouse brain and reduces pulsatile GnRH secretion in female pubertal monkeys (43), underscoring the importance of kisspeptin signaling in the control of GnRH secretion.

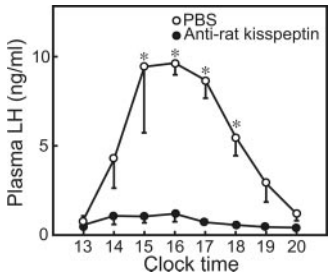

FIG. 3. Effect of POA infusion of antirat kisspeptin monoclonal antibody on the proestrous LH surge. PBS (n = 6) or antirat kisspeptin monoclonal antibody (n = 5) was infused into the POA at 1000–1800 h. Blood samples were collected every hour at 1300–2000 h through an indwelling atrial cannula. Values are means ± SEM. *, $P < 0.05$ *vs.* vehicle-treated control (one-way ANOVA with repeated measures). [Modified with permission from Kinoshita *et al.*, 2005 (42) © The Endocrine Society].

IV. Comparative Anatomy

The distribution and physiology of kisspeptin-Kiss1r signaling has been explored in a variety of species. Although the location and developmental timing and patterns of expression differ among species, this pathway clearly plays an important role in reproduction in many vertebrates; however, given their diversity, the extraordinary range of reproductive strategies, and our incomplete knowledge of the functional significance of kisspeptin signaling across these species, it is difficult (and hazardous) to draw unifying themes. Nevertheless, a current comprehensive description of the development, localization, and sexual differentiation of kisspeptin and its receptor for all species studied to date (summer 2009) follows.

A. Nonmammalian vertebrates

The anatomy and physiology of Kiss1/Kiss1r signaling in fish has recently been reviewed (44, 45). A variety of piscine species have been studied; however, a unified synopsis of kisspeptin physiology in fish is complicated by several factors. First, there is an astonishing diversity of reproductive strategies among species of fish (*i.e.*, semelparous, iteroparous, hermaphroditic species). Second, fish occupy a vast range of environmental niches—much wider and more diverse than mammals. Third, the time to sexual maturity varies considerably across species; and fourth, investigators studying fish have taken different experimental approaches to investigate kisspeptin biology. Most information collected on the kisspeptin system in fish consists of work exploring the receptor, Kiss1r. Indeed, it was not until 2008 that information on kisspeptin neurons was reported in nonmammalian vertebrates. Details on developmental expression, localization, and sexual differentiation of Kiss1 and Kiss1r in piscine species studied to date are summarized below.

1. Tilapia (Oreochromis niloticus)

Parhar *et al.* (46) were the first to identify Kiss1r in any piscine species and to report colocalization of Kiss1r and GnRH expression in neurons of the tilapia. It is notable that this was the first report of colocalization of Kiss1r and GnRH neurons in any species— an early benchmark establishing that GnRH neurons are direct targets for the action of kisspeptin. Utilizing a single-cell gene profiling (laser-captured microdissection) approach, these investigators demonstrated *Kiss1r* transcript expression in all three teleost GnRH neuronal types: GnRH-I (POA), GnRH-II (midbrain tegmentum), and GnRH-III (caudalmost part of the olfactory bulbs) (46). The POA GnRH-I is thought to control the synthesis and release of LH and FSH in most vertebrate species, and GnRH-I neurons have been shown to innervate the anterior pituitary in teleosts (47). Conspicuously, the developmental pattern of Kiss1r expression in tilapia appears to correspond with that of the GnRH-I receptor, both of which display an increase between 3–4 and 6–7 wk after hatch, corresponding with the onset of puberty (48). Assuming translation into increased Kiss1r protein, an increase in *Kiss1r* expression in GnRH cells could plausibly increase the responsiveness of GnRH neurons to kisspeptin, thus contributing to the increase in GnRH secretion associated with the onset of puberty. Although no sex differences were identified in *Kiss1r* expression in the brain, higher levels of *Kiss1r* expression were observed in the pituitary of females (48). The expression of *Kiss1r* was also evaluated in the heart, kidney, liver, gonad, and muscle, but expression was near the limits of detection in these tissues (48).

2. Gray mullet (Mugil cephalus)

Mullet Kiss1r shares a 95% sequence homology with the tilapia receptor and is expressed in the brain, pituitary, and ovary. In this species, just as in the tilapia, Kiss1r may play a role in reproductive development, based on the observation that mullet *Kiss1r* is induced in the brain just at the onset of puberty (49). Moreover, the pattern of *Kiss1r* gene expression is positively correlated with that of GnRH-II and GnRH-III (49), suggesting coordinated roles of Kiss1r and GnRH in the brain at the early stages of puberty. At more advanced stages of reproductive development, levels of the *Kiss1r* transcript in the ovary are increased compared with expression levels in the brain (49), suggesting a supporting role of the Kiss1/Kiss1r signaling in gonadal development, as well as in the neuroendocrine axis.

3. Cobia (Rachycentron canadum)

In the brain of the cobia, the expression of *Kiss1r* mRNA peaks at 26 d after hatching, during the juvenile stage (47). Notably, the pattern of *Kiss1r* expression and

all three *GnRH* mRNAs (*GnRH*-I, -II, -III) is remarkably similar throughout the early larval and juvenile periods of development (47). Again, these findings point to a potential relationship between Kiss1r and multiple GnRHs and implicate Kiss1r in the development and maturation of the reproductive system in piscine species.

4. Senegalese sole (Solea senegalensis)

Analysis of the Senegalese sole (Ss) Kiss1r by Mechaly *et al.* (50) revealed features indicative of alternative splicing. RT-PCR identified two distinct transcripts differentiated by approximately 80 bp in length and named Ss Kiss1r_v1 (or short Ss Kiss1r) and Ss Kiss1r_v2 (or long Ss Kiss1r). The two isoforms exhibit differential patterns of expression in various tissues. In the brain, levels of *Ss Kiss1r_v1* mRNA are higher than those of *Ss Kiss1r_v2*, whereas in the gonads, the predominant isoform is *Ss Kiss1r_v2* (50). Outside of the brain, both isoforms are expressed in the testis, liver, muscle, stomach, heart, spleen, and kidney, whereas the ovary and gall bladder express only the *Ss Kiss1r_v2* isoform, and the intestine expresses only the *Ss Kiss1r_v1* isoform (50). However, both isoforms exhibit changes in expression as a function of sex and maturational stage. For example, in the brain, levels of the mRNAs of both isoforms are higher in the pubertal compared with mature female sole (50), suggesting that kisspeptin signaling may play a "gatekeeper" role for timing the onset of puberty in this as well as other piscine species.

5. Fathead minnow (Pimephales promelas)

In the adult fathead minnow, *Kiss1r* mRNA is expressed throughout the brain and in the pituitary and gonad, but it is undetectable in muscle, intestine, liver, or gill (51). In the brain, *Kiss1r* is expressed predominantly in the telencephalon (including the POA); moderately in the olfactory bulbs and tracts, optic tectum, and hypothalamus/midbrain tegmentum; and at low levels in the optic nerves, medulla oblongata, and cerebellum (51). Sexual dimorphism in the expression of *Kiss1r* is not evident in the whole brain. In the brain, the highest expression of Kiss1r occurs in regions where the *GnRH* genes are highly expressed (51). Moreover, the developmental pattern of *Kiss1r* expression closely aligns with the expression of GnRH-III, the hypophysiotropic form of GnRH in the fathead minnow (which, like other cyprinids, does not seem to express GnRH-I) (51). Neural *Kiss1r* expression increases at the onset of puberty in both male and female fathead minnows; moreover, these high levels correspond with the appearance of spermatogonia in the testis in males and of cortical alveolus stage oocytes in the ovary in females. The expression of *Kiss1r* in the brain is 4-fold higher in sexually mature females compared with prepubertal females (51). Furthermore, injections of mammalian kisspeptin-10 into early to midpubertal fish induced expression of GnRH-III and Kiss1r in the brain, suggesting an autoregulatory effect of kisspeptin on its own receptor (51).

6. Medaka (Oryzias latipes)

The kisspeptin receptor, Kiss1r, has been characterized in several piscine species, as detailed in the preceding sections; however, it was not until 2008 that kisspeptin neurons were studied in nonmammalian vertebrates. Utilizing RT-PCR, Kanda *et al.* (52) revealed the expression of *Kiss1* mRNA in the brain, testis, and stomach (and its absence in the ovary, liver, intestine, and retina) of the medaka. *In situ* hybridization identified two distinct hypothalamic nuclei that contain Kiss1-expressing neuronal cell bodies: the nucleus posterioris periventricularis and the nucleus ventral tuberis (NVT) (52). The NVT Kiss1 neurons are sexually dimorphic in number (male neurons $\gg$ female neurons) and steroid sensitive, whereas nucleus posterioris periventricularis neurons are neither (52). For instance, estrogen treatment rescues the ovariectomy-initiated decrease in Kiss1-expressing neurons of the NVT (52), indicating the necessity of the sex steroids for the maintenance of Kiss1 expression. This research group suggests that the Kiss1/Kiss1r system plays a role in triggering the onset of puberty, based on the preliminary demonstration that neural Kiss1 and Kiss1r increase dramatically during sexual maturation in this species (53).

Recent work by Parhar and colleagues (54) has identified a gene similar to the *Kiss1* gene identified by Kanda. This novel kisspeptin gene, an RFamide (as distinguished from an RY-amide), has been named *Kiss2* (Fig. 4). Medaka *Kiss2* is expressed in the brain (periventricular hypothalamus), testis, ovary, intestine, kidney, and heart (54), hinting at a role in reproductive as well as nonreproductive processes. Moreover, *Kiss1* mRNA-containing cells are found in the ventromedial region of the habenula and in the posterior tuberal nucleus zone of the periventricular hypothalamus (54), where their physiological significance has yet to be revealed.

7. Goldfish (Carassius auratus)

Recent work by Li *et al.* reveals that goldfish express *Kiss1* and *Kiss2* as well as their putative cognate receptors, *Kiss1ra* and *Kiss1rb* (55). The *Kiss1* gene is highly expressed in the optic tectum thalamus, intestine, kidney, and testis, whereas the *Kiss2* gene is mainly detected in the hypothalamus, telencephalon, optic tectum, thalamus, adipose tissue, kidney, heart, and gonads. The two receptor genes (*Kiss1ra* and *Kiss1rb*) are highly expressed in brain

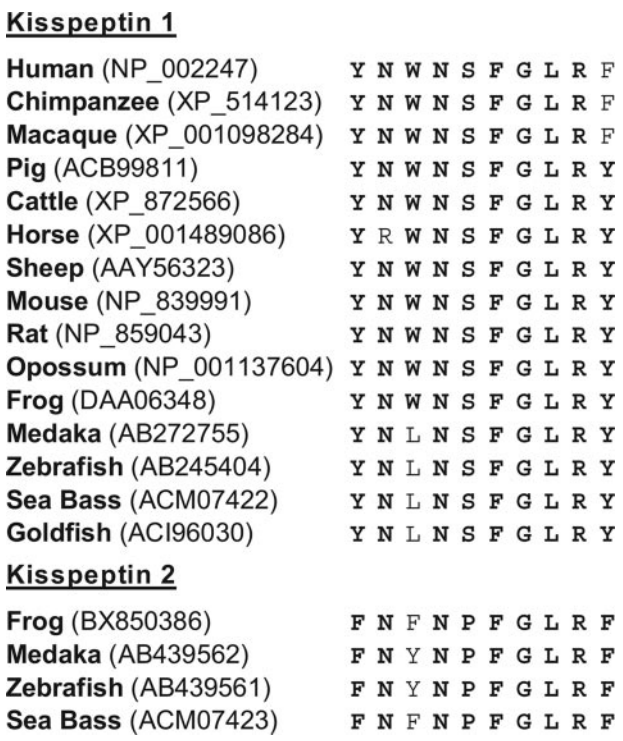

FIG. 4. Kisspeptin-10 sequences. Alignment of deduced amino acid sequences (from BLAST) of *Kiss1* and *Kiss2* in vertebrates. Conserved amino acid residues are in *bold*.

regions including telencephalon, optic tectum, thalamus, and hypothalamus, as well as in peripheral tissue, including the gonads and adipose tissue; additionally, *Kiss1rb* is expressed in liver, intestine, gill, heart, and kidney (55). Furthermore, both mature goldfish kisspeptin-10 peptides (Kiss1–10 and Kiss2–10) can functionally interact with the two receptors expressed in cultured cells, indicating that they are biologically active (55). Utilizing RT-PCR coupled to laser capture microdissection, Yang *et al.* (56) found *Kiss1r* expression in gonadotrophs, somatotrophs, and lactotrophs of the goldfish pituitary, as well as *Kiss1* expression in somatotrophs. Moreover, incubation with kisspeptin-10 increases basal levels of LH, GH, and PRL mRNA in goldfish pituitary cells, suggesting a direct action of kisspeptin at the level of the pituitary.

8. Zebrafish (Danio rerio)

Two different kisspeptin genes (*Kiss1* and *Kiss2*), as well as two distinct kisspeptin receptors (*Kiss1ra* and *Kiss1rb*), have been characterized in the zebrafish. The two receptors differ in their tissue expression distribution; they are sexually dimorphic, and they signal through unique transduction pathways. They share approximately 60% sequence identity with each other, with Kiss1ra being more similar to other piscine Kiss1r (91% identity) and Kiss1rb being more like mammalian Kiss1r (57). Both zebrafish kisspeptin receptors are highly expressed in the brain. *Kiss1ra* is also expressed at high levels in the gonads (testis ≫ ovary), and *Kiss1rb* is highly expressed in the pituitary, spleen, gills, kidney, intestines, pancreas, and adipose tissue (57). A role in puberty onset is suggested by real-time PCR analysis, which revealed increasing levels of *Kiss1ra* mRNA in the brain of both male and female zebrafish (females ≫ males) until the age when gonads contain well-developed oocytes and spermatozoa (57). Recent work has begun to explore the pathways through which these receptors signal. Using serum-responsive element-luc and cAMP-responsive element-luc reporter systems to follow protein kinase C (PKC) and protein kinase A pathway activation, Biran *et al.* (57) demonstrated that zebrafish *Kiss1ra* transduces its activity via the PKC pathway, whereas *Kiss1rb* does so via both PKC and protein kinase A pathways. Zebrafish *Kiss1ra* and *Kiss1rb* are both highly expressed in the hindbrain. *Kiss1ra* is moderately expressed in the telencephalon, and *Kiss1rb* is moderately expressed in the diencephalon and midbrain (57). The neuronal circuitry linking the expression of kisspeptin to its target cells and receptors has yet to be fully elucidated (in any species).

Both zebrafish *Kiss1* and *Kiss2* are expressed in the brain (*Kiss1* in the ventromedial region of the habenula, and *Kiss2* in the posterior tuberal nucleus and the periventricular hypothalamus), testis, and intestine, whereas *Kiss1* is also expressed in the pituitary, adipose tissue, pancreas, heart, and liver, and *Kiss2* in the ovary and kidney (54, 57, 58). Zebrafish *Kiss1*, *Kiss2*, *GnRH-II*, and *GnRH-III* mRNA levels all show an increase in expression at the start of the pubertal phase (54), demonstrative of a potential role in controlling the onset of puberty. It is notable that ip injections of Kiss2 decapeptide (but not Kiss1) into sexually mature female zebrafish activates gonadotropin gene expression ($lh\beta$ and $fsh\beta$) in the pituitary (54), implicating Kiss2 as the principal regulator of gonadotropin synthesis and thus a powerful regulator of reproduction.

9. Sea bass (Dicentrarchus labrax)

Reports of a Kiss1/Kiss1r system have recently been made in the European sea bass. Results indicate the expression of two *Kiss1r* and two *Kiss1*-like genes. Preliminary data suggest that both *Kiss1r* genes are expressed largely in the brain, pituitary, testis, and ovary and less so in the spleen, kidney, liver, intestine, gill, heart, eye, skin, and muscle of prepubertal and pubertal male and female sea bass (59). Both kisspeptin genes (*Kiss1* and *Kiss2*) are expressed principally in the brain and gonadal tissues of pubertal sea bass and do not appear to demonstrate developmental stage or sex specificity (60, 61). Intramuscular injections of both Kiss1 and Kiss2 stimulate gonadotropin secretion in prepubertal sea bass (preliminary data), suggesting that the Kiss1/Kiss1r system is involved in pu-

bertal development (60, 62). Exciting new work by Carrillo and colleagues (63) has begun to identify the distribution of the sea bass kisspeptin-immunoreactive (ir) system through the use of rabbit antibodies against mouse Kiss-10. Their recent studies suggest that the kisspeptin expression is prominent in the nucleus posterioris periventricularis and projects to a variety of areas of the brain, including the thalamic region, the midbrain tegmentum, and the pituitary stalk. Furthermore, Kiss1 fibers appear to appose GnRH-II neurons in the midbrain tegmentum, as demonstrated via preliminary double-staining (63). This pioneering work with Kiss1 protein localization in a piscine species provides a novel technique that still needs to be used in other fish species.

10. Xenopus tropicalis and laevis (X. tropicalis has now been reclassified as Silurana tropicalis)

Xenopus express three isoforms of kisspeptin genes: *Kiss1a*, *Kiss1b*, and *Kiss2* and three forms of receptors: *Kiss1ra*, *Kiss1rb*, and *Kiss1r2* (64). All types of kisspeptin and *Kiss1r* mRNAs are expressed in the hypothalamus of *X. tropicalis* (64). In addition, *Kiss1a* mRNA is expressed in most tissues except the oocytes; *Kiss1b* mRNA is observed in the forebrain, hindbrain, testis, heart, lung, intestine, and eye; and *Kiss2* mRNA is expressed in the testis, heart, kidney, and liver. *Kiss1ra* mRNA is found in the forebrain, pituitary, testis, and intestine; *Kiss1rb* mRNA is expressed in the forebrain, hindbrain, testis, and liver; and *Kiss1r2* mRNA is found in the forebrain, pituitary, and heart (64). In *X. laevis*, *Kiss1* mRNA is expressed in the ventral hypothalamus (VH) and *Kiss2* in the POA and VH. Kiss2 immunoreactive cells bodies are also restricted to the POA and VH, with fibers terminating in the median eminence (ME)—suggesting that Kiss2 peptide may regulate GnRH release presynaptically at the level of the ME or be released to the pituitary through the hypothalamo-pituitary portal system.

11. Bullfrog (Rana catesbeiana)

The bullfrog kisspeptin receptor, *Kiss1r*, has recently been isolated, and expression has been found in the forebrain, hypothalamus, and pituitary, with weak expression in the testis and no detectable expression in the adrenal gland, heart, kidney, lung, ovary, spleen, and stomach (65).

12. Birds (zebra finch, Taeniopygia guttata)

Tobari *et al.* (66) have recently described the distribution of kisspeptin peptide in the brain of the adult male zebra finch. Using immunohistochemistry, the authors observed preliminary kisspeptin-like-immunoreactive (Kiss1-like-ir) cells in the nucleus infundibularis, which is homologous to the mammalian ARC and receives neural inputs from song control and auditory brain regions. Kiss1-like-ir fibers and terminals are present in hypothalamic nuclei (*i.e.*, nucleus periventricularis magnocellularis, nucleus preopticus anterioris, and medialis), the telencephalon, mesencephalon, and medulla (including the pars tracheosyringealis, which controls the avian vocal organ) (66). Together, these preliminary observations support a role of kisspeptin in regulation of not only GnRH release, but also song control in the adult male zebra finch.

13. Other nonmammalian vertebrates

A comparative genomics approach has identified putative kisspeptin sequences in the genomes of various nonmammalian vertebrates including: fugu (*Takifugu rubripes*), tetraodon (*Tetraodon nigroviridis*), sea lamprey (*Petromyzon marinus*), three-spined stickleback (*Gasterosteus aculeatus*), elephant shark (*Callorhinchus milii*), and frog (*Xenopus laevis/tropicalis*) (50, 54, 57, 58, 65, 67). Investigations of these other fish, avian, reptilian, and amphibian species may reveal more surprises and hitherto unexpected roles for kisspeptin signaling.

B. Mammals

A comparative genomics approach has also yielded putative kisspeptin sequences in the genomes of a wide variety of mammalian species, such as opossum (*Monodelphis domestica*) (57), lesser hedgehog (*Echinops telfair*) (58), and even platypus (54), although the majority of research on the kisspeptin signaling system in mammals has focused on "traditional" research species, such as rodents, livestock, and primates.

1. Rodents

The relative ease with which the genetic composition of mice can be experimentally manipulated makes them an ideal experimental animal for the study of kisspeptin signaling and understanding of the role of kisspeptin in reproductive neuroendocrinology. Other rodent models also offer unique advantages as well. We understand a great deal about the physiology of the laboratory rat; moreover, these animals have a relatively large blood volume (for a rodent), which can be readily sampled for hormone measurements. Hamsters have the virtue of being highly seasonal, which offers a special window on aspects of circadian physiology and photoperiodic signaling not afforded by other nonseasonal animals, such as the laboratory mouse and rat.

a. Mouse (Mus musculus). Studies in the mouse have provided a strong foundation for our understanding of kisspeptin signaling in the mammalian brain. For instance, mutant mice with a targeted disruption of *Kiss1r* provide

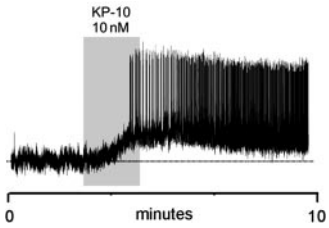

FIG. 5. Kisspeptin exerts a potent activational effect on GnRH neurons in adult female proestrous mice. Perforated-patch, voltage recordings from proestrous female GnRH-GFP neurons (resting membrane potential = −68 mV) in the acute brain slice demonstrate a remarkably intense and prolonged activation by 10 nM kisspeptin (KP-10). [Modified with permission from Han *et al.*, 2005 (70)].

evidence that Kiss1r is essential for the development of the murine reproductive system (9, 68, 69). Notwithstanding the progress in our understanding of kisspeptin biology, it is astonishing that a detailed map of Kiss1r expression in the murine brain has yet to be published. Nevertheless, it has been shown that *Kiss1r* is expressed in GnRH neurons (70), establishing that these cells are almost certainly direct targets for kisspeptin action. Through the use of a lacZ reporter in mutant *Kiss1r*$^{-/-}$ mice, β-galactosidase activity (a marker for Kiss1r expression) is evident in approximately 55% of GnRH-ir neuronal cell bodies located in the preoptic area of the hypothalamus (71). Utilizing dual-label *in situ* hybridization for *GnRH* and *Kiss1r* mRNA, Han *et al.* (70) found that more than 90% of GnRH neurons express *Kiss1r* transcript, thus providing evidence that, in the mouse, kisspeptin neurons provide direct synaptic input to GnRH neurons, an idea corroborated by the finding that kisspeptin exerts a potent, direct depolarizing action on GnRH neurons (70) (Fig. 5).

A detailed distribution of Kiss1 (transcript and protein) has been mapped in the murine hypothalamus. In this species, *Kiss1* mRNA and Kiss1-immunoreactive cell bodies are expressed in areas of the hypothalamus implicated in the neuroendocrine regulation of gonadotropin secretion, including the anteroventral periventricular nucleus (AVPV), the periventricular nucleus (PeN), and the ARC (72, 73) (Fig. 6). In addition, some cells expressing *Kiss1* mRNA are located in the anterodorsal preoptic nucleus, a few cells are found in the medial amygdala and bed nucleus of the stria terminalis, and none are present in the caudate nucleus, globus pallidus, nucleus accumbens, putamen, and striatum (72). Using *Kiss1* knockout (and wild-type) mice, Clarkson *et al.* (74) have recently published a comprehensive map of the distribution of Kiss1-ir cells in the mouse, which has helped to clarify some earlier confusion related to the nonspecificity of kisspeptin antibodies. Generally, the pattern of kisspeptin cell body distribution overlaps remarkably well with that described for *Kiss1* mRNA in the mouse, with only a few discrepancies. Immunocytochemical studies have revealed two dense populations of

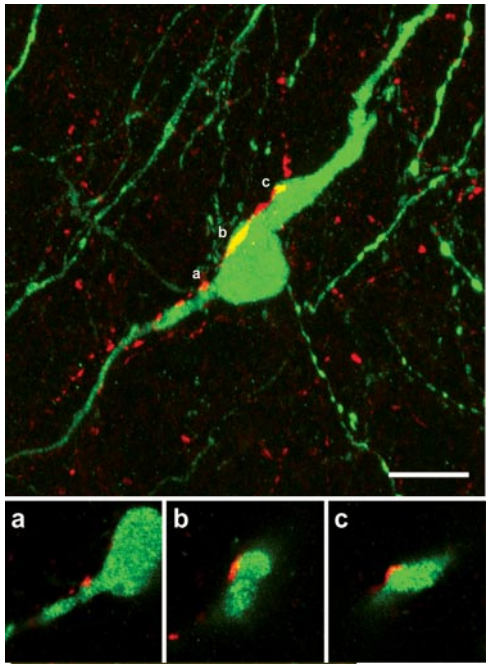

FIG. 6. Kisspeptin projections to GnRH neurons in adult female mice. Confocal stack of 75 images showing a single GnRH neuron (*green*) with kisspeptin (*red*) fibers surrounding and apposed to it. Single 370-nm-thick optical sections through the three regions indicated by a, b, and c of the GnRH neuron are given below to demonstrate the close apposition between kisspeptin fibers and GnRH neuron elements. Scale bar, 10 μm. [Modified with permission from Clarkson and Herbison, 2006 (73) © The Endocrine Society].

Kiss1-ir cell bodies— one in the rostral continuum of the third ventricle (including the AVPV and PeN), and another in the ARC. Less-dense and more scattered populations of Kiss1-ir cell bodies have also been identified in the dorsomedial nucleus and posterior hypothalamus; moreover, dense concentrations of Kiss1-ir fibers are found within the ventral aspect of the lateral septum and along periventricular and ventral retrochiasmatic pathways, with scattered fibers appearing in the bed nucleus of the stria terminalis, medial amygdala, subfornical organ, paraventricular thalamic nucleus, the supraoptic and paraventricular nuclei, as well as the periaqueductal gray and locus coeruleus. Kiss1-ir fibers are absent from the ventromedial hypothalamic nucleus (VMH) and the suprachiasmatic nucleus (73, 74). Although the overall distribution of Kiss1-ir cells is similar between male and female mice, there is a remarkable sex difference in the number of cell bodies in the AVPV/PeN [as is the case with *Kiss1* mRNA-expressing cells in the rat (75)], with adult females exhibiting 10-fold greater numbers of kisspeptin-ir cells than males (73). (It should be noted that the specificity of the antiserum used for some of the early immunocytochemical studies was not properly validated, and thus some of the results may reflect nonspecific labeling).

b. Hamster (Syrian, Mesocricetus auratus; and Siberian, Phodopus sungorus). Seasonally breeding rodents, such as the "long-day" breeding hamster, are useful to study the effects of photoperiod on reproductive function. Although there is no information currently available about Kiss1r localization in the hamster, several groups have reported on the distribution of Kiss1 expression (both mRNA and peptide). Revel *et al.* (76) reported expression of both *Kiss1* mRNA and peptide product in the ARC of Syrian hamsters raised in long-day photoperiod. In the Syrian hamster, no Kiss1-ir cell bodies could be found in the AVPV (76), but in the Siberian hamster, Kiss1-ir cells have been observed in both the AVPV and the ARC, which may reflect either differences between species or the efficacy of the antiserum and/or methodology (77, 78).

c. Rat (Rattus norvegicus). Although the regulation of *Kiss1* expression in the ARC, AVPV, and PeN of the rat has been extensively investigated by *in situ* hybridization (75, 79–82), a thorough description of the distribution of *Kiss1* mRNA-expressing cells throughout the brain has not been published. The results of a broad screen for *Kiss1* mRNA by RT-PCR suggested that the *Kiss1* gene is expressed throughout the rat central nervous system, including the spinal cord, medulla and pons, midbrain, hypothalamus, and cerebral cortex, with the highest concentrations occurring in the hypothalamus, midbrain, and spinal cord (83). A more detailed description of distribution of Kiss1 neurons in the rat brain has been obtained by immunohistochemistry. Antiserum directed against human KISS-45-54 labeled Kiss1-ir neurons in the ARC, DMH, paraventricular nuclei, VMH, caudoventrolateral reticular nucleus, lateral reticular nucleus, nucleus of the solitary tract, and spinal trigeminal tract (83, 84). Additionally, Kiss1-ir cell processes were found in many other locations, including the nucleus accumbens, amygdala, thalamus, hypothalamus, bed nucleus of stria terminalis, septal nuclei, nucleus accumbens, caudate putamen, diagonal band of broca (DBB), amygdala, zona incerta, thalamus, periaqueductal gray, raphe nuclei, lateral parabrachial nucleus, locus coeruleus, spinal trigeminal tract, rostral ventrolateral medulla, and medullary reticular nucleus (83). In particular, Kiss1-ir fibers were described in the medial preoptic area, anterior hypothalamic area, paraventricular nucleus, and ARC (83), areas of known importance in the control and regulation of gonadotropin secretion. Unfortunately, discrepancies between *in situ* hybridization results and the results of immunohistochemical studies that utilize the human KISS1-45-54 antibody raise concerns about the specificity and sensitivity of that antibody. The greatest numbers of Kiss1-ir cells in the hypothalamus were found in the DMH, but cells containing *Kiss1* mRNA cannot be detected in the DMH by *in situ* hybridization. Furthermore, the antibody did not label Kiss1 neurons in the AVPV/PeN, a region in which the expression of *Kiss1* mRNA has been well established by *in situ* hybridization. It should also be borne in mind that the rat is similar to other species in that the expression of *Kiss1* is sexually differentiated in some areas of the brain (73, 75). This is particularly true in the AVPV, where there are about 25 times more Kiss1 cells in adult females compared with males (75).

The presence of *Kiss1r* in the brain was first reported in 1999 by Lee *et al.* (6), who used Northern blot and *in situ* hybridization and found expression in the pons, midbrain, thalamus, hypothalamus, hippocampus, amygdala, cortex, frontal cortex, and striatum, as well as peripheral organs such as the liver and intestine; no *Kiss1r* message was found in the cerebellum or kidney. A more detailed examination of *Kiss1r* transcript in the forebrain has revealed expression in the DBB, medial septum, medial preoptic area, lateral preoptic area, median preoptic nucleus, anterior hypothalamus, and lateral hypothalamus (80). To determine whether kisspeptin acts directly on GnRH neurons, Irwig *et al.* (80) used double-label *in situ* hybridization to discover that more than 75% of GnRH neurons coexpress *Kiss1r* mRNA (Fig. 7). In addition, a recent preliminary report indicates that Kiss1-ir fibers terminate in close proximity to GnRH fibers in the ME (as shown via electron microscopy), and kisspeptin causes GnRH release from ME rat tissue *in vitro* (85). A role of kisspeptin in reproductive neuroendocrine signaling outside of the brain is suggested by the finding that both Kiss1 and Kiss1r are expressed in pituitary gonadotrophs (LHβ-ir cells), as demonstrated by dual immunofluorescence (86). Thus,

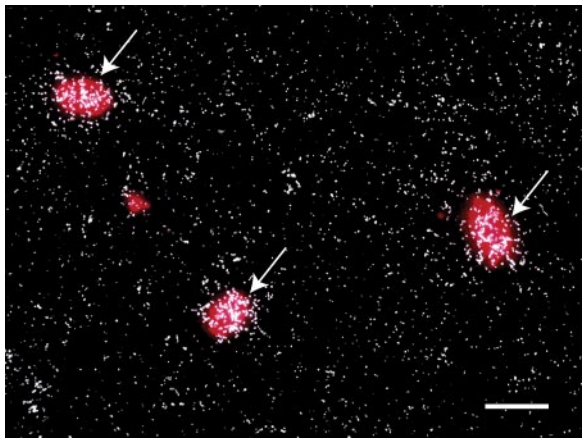

FIG. 7. Coexpression of GnRH mRNA with *Kiss1* mRNA in rats. Representative photomicrograph from the medial preoptic area. GnRH-mRNA-expressing cells are fluorescent with Vector Red substrate. Clusters of silver grains (*white dots*) reflect the presence of *Kiss1r* mRNA. The *arrows* indicate GnRH neurons that coexpress *Kiss1r*. Approximately 77% of the GnRH neurons coexpress *Kiss1r* mRNA (n = 4 rats). Scale bar, 20 μm. [Reproduced with permission from Irwig *et al.*, 2004 (80) © S. Karger AG].

kisspeptin may regulate reproductive function at both the hypothalamic and pituitary level.

2. Ungulates

Large, hoofed farm mammals are an important economic resource, and they share many physiological characteristics with humans—and thus, have been a target of research in reproduction. Because of their large size and gentle demeanor, ungulates readily tolerate serial blood sampling to monitor hormone levels, and they offer ready access to the pituitary portal blood for the measurement of hypophysiotropic hormones (87). Knowledge about the neuroendocrine regulation of gonadotropin secretion in such animals could provide insight into how to synchronize or predict ovulation in humans, which could be useful for the treatment of infertility; moreover, such information could also be useful for the development of strategies to accelerate growth and puberty onset in these domestic species, which greatly improves the economics of commercial breeding of meat-producing species.

a. Goat (Capra hircus). A recent study in the goat suggests that kisspeptin signaling plays a vital role in the pulsatile secretion of GnRH and LH. In this species, Kiss1-ir neurons and fibers are clustered in the posterior ARC, with dense fibers appearing in the zona interna of the ME (88). Electrical recordings of multiple unit activity and simultaneous measurements of plasma levels of LH show remarkable coincidence in their ultradian profiles, suggesting that kisspeptin neurons in the ARC may be the proximate source of GnRH pulse generator activity (88)—an idea reinforced by observations in the monkey showing nearly coincident secretion of kisspeptin and GnRH in the medial basal hypothalamus (MBH) (89).

b. Pig (Sus domestica). The kisspeptin system has only recently been explored in the pig. Semiquantitative RT-PCR has identified abundant *Kiss1r* transcript in the adrenal, prostate, testis, thymus, pituitary, and hypothalamus, with weak expression in the heart and lung (90). Real-time quantitative RT-PCR of *Kiss1r* mRNA content in the hypothalamus reveals fluctuating levels throughout the estrous cycle, with lower expression levels during the follicular phase and the highest level occurring in the luteal phase (90). In comparison to cyclic sows, juvenile (anestrous) animals exhibit markedly lower hypothalamic *Kiss1r* transcript expression (90), a finding consistent with a possible role of kisspeptin in initiating puberty.

c. Sheep (Ovis aries). The ARC serves as the predominant locus for the expression of Kiss1-ir in the hypothalamus of the sheep. Additionally, Kiss1-ir is observed in the DMH, the medial preoptic area, the PeN, VMH, and the caudal region of the paraventricular nucleus (91, 92). The highest density of Kiss1-ir varicose nerve fibers is found in the ME and the POA, as well as the ARC, PeN, VMH, and DMH (91, 92). Kiss1-ir is detected in the POA, an area known to contain numerous GnRH neurons in sheep (93). Pompolo *et al.* (91) examined whether kisspeptin colocalizes with GnRH neurons. In an initial study, they reported that GnRH cells in the DBB/POA and GnRH neurosecretory terminals of the ME contain kisspeptin-ir; however, it now appears that this finding reflects the nonspecificity of the antibody used to detect kisspeptin (from Phoenix Pharmaceuticals) (94). Using a kisspeptin-specific antibody (from Alain Caraty at the University of Tours, Tours, France) (92), Smith *et al.* (94) now report that GnRH neurons in sheep do not coexpress kisspeptin. In any case, the functional significance of Kiss1 expression in the medial POA of the sheep remains to be determined. It also appears that *Kiss1r* mRNA is expressed in ovine pituitary cell fractions enriched for gonadotropes, and low, but detectable, amounts of Kiss1-ir are found in hypophyseal portal blood, which leaves open the possibility that kisspeptin is either a hypophysiotropic/neurosecretory factor in sheep or that there are paracrine mechanisms involving kisspeptin-Kiss1r signaling within the pituitary itself (95).

d. Horse (Equus caballus). The size and distribution of Kiss1-ir cell bodies in the ARC of the Welsh pony mare (collected just after ovulation) are similar to those in the sheep (92), with additional cells localized to the DMH (96). Additionally, a scattering of Kiss1-ir cells in the horse are localized to the preoptic periventricular zone of the hypothalamus adjacent to the third ventricle. Again, there is some concern about the specificity of the antibodies used to detect kisspeptin in these reports, with possible cross-reactivity with other RFamide peptides. Kiss1-ir varicose fibers are evident from the POA to the mammillary nuclei, with a high density present in the anterior periventricular area and the ME (96, 97). Immunoreactive Kiss1 neurons are not observed in the POA; moreover, kisspeptin is not colocalized in GnRH neurons—which is consistent with the revised analysis in sheep (94, 96). However, close appositions between Kiss1-ir fibers and GnRH neurons are evident in the periventricular area, the ARC, and the ME, as analyzed by confocal microscopy (96, 97). Collectively, these results suggest that kisspeptin is involved in regulation of GnRH release at GnRH neuronal terminals at the time of the LH surge in Welsh pony mares.

3. Primates

The discovery that disabling mutations in the kisspeptin receptor is responsible for producing a form of hypogonadotropic hypogonadism in humans established that kisspeptin signaling is a critical feature in the devel-

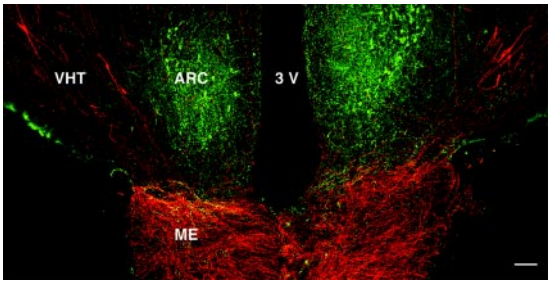

FIG. 8. Structural interactions between Kiss1 and GnRH neurons in the mediobasal hypothalamus of the male rhesus monkey. A confocal projection (×10; 1-μm optical sections) illustrating the distribution of kisspeptin neurons (green fluorescence, Alexa Fluor 488) in relation to the GnRH neuronal network (red fluorescence, Cy3) in a coronal section of the MBH of an agonadal male rhesus monkey aged 4 yr 3 months. Whereas kisspeptin perikarya were confined to the ARC, those of GnRH extended along the ventral hypothalamic tract (VHT), lateral to the ARC. GnRH innervation of the external zone of the ME was intense. Beaded kisspeptin axons projected to the ME, and at this anteroposterior level GnRH and kisspeptin fibers running in a near horizontal plane were found in close association. 3V, Third ventricle. Scale bar, 100 μm. [Modified with permission from Ramaswamy *et al.*, 2008 (99) © The Endocrine Society].

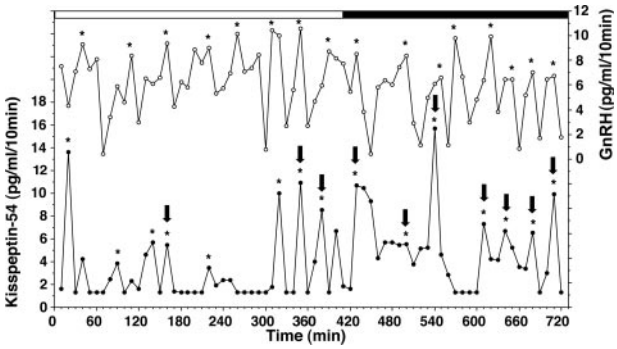

FIG. 9. Kisspeptin-54 release in the stalk-ME of the monkey, as assessed by microdialysis, is pulsatile. Both kisspeptin-54 (*closed circle*) and GnRH (*open circle*) were measured in the same microdialysate samples. Kisspeptin-54 and GnRH pulses, indicated by *asterisks*, were identified using the PULSAR algorithm. Kisspeptin-54 pulses correlated with GnRH pulses are indicated by *arrows* on the top of kisspeptin pulses. Lighting conditions are indicated on the top of each graph (*white bar* for the lights-on period and *black bar* for the lights-off period). [Modified with permission from Keen *et al.*, 2008 (89) © The Endocrine Society].

opment and control of reproduction (8, 98). Performing studies in humans is often of limited scope (or precluded), based on ethical considerations; however, research performed in the nonhuman primate species, while also restricted under careful ethical guidelines, has offered some global insight into the anatomy and physiology of kisspeptin and its receptor in reproduction in primates in general, including humans.

a. Rhesus macaque (Macaca mulatta). In the rhesus monkey, Kiss1-ir perikarya have been identified in the ARC/ME, but not in the POA (including the AVPV) (99, 100); moreover, kiss1-ir is not identifiable in GnRH perikarya (99). Kiss1-ir projections are detected throughout the MBH and to a lesser extent in the POA (99), which complements the finding of *Kiss1r* in these hypothalamic areas (100, 101). Kiss1-ir axons make only infrequent contacts with GnRH cell bodies in the MBH (25–50%) (Fig. 8). However, in the ME (internal zone and to a lesser extent external layer), kisspeptin and GnRH axons are extensively and intimately associated, although only occasional axo-axonal contacts between the two have been identified (99). Nevertheless, the dual innervation of the ME by both kisspeptin and GnRH suggests that kisspeptin may regulate GnRH secretion nonsynaptically at the level of the ME. Further evidence that this might be the case comes from the observation that kisspeptin is released into the ME of the monkey in a pulsatile fashion that is synchronized with the pulsatile release of GnRH in the same region (89) (Fig. 9).

b. Human (Homo sapiens). Studies of families with idiopathic hypogonadotropic hypogonadism pointed to a mutation in *KISS1R*, suggesting that this receptor is essential for normal GnRH secretion and commencement of puberty. In one of the earliest papers on kisspeptin, Kotani *et al.* (2) reported abundant expression of *KISS1R* transcript (via RT-PCR) in human placenta, pituitary, spinal cord, and pancreas, with lower levels evident in other tissue, such as various brain regions, stomach, small intestine, thymus, spleen, lung, testis, kidney, and fetal liver. Transcript for the receptor's ligand (*KISS1* mRNA) has been identified in human placenta, testis, pancreas, liver, and small intestine (4). Within the hypothalamus, KISS1 neurons are present predominantly in the infundibular nucleus (which is the homolog of the ARC in rodents and some other animals, including sheep) and in sparse and indiscrete foci in the medial preoptic area (102). No kisspeptin neurons have been reported in the rostral periventricular region of the third ventricle; however, so far the distribution of KISS1 cell bodies has only been described in sagittal brain slices, in which periventricular cells are difficult to observe. Recently, Hrabovszky *et al.* (103) have created a preliminary detailed anatomical map of KISS1-ir fibers in the human hypothalamus. Axon terminals with kisspeptin-like-ir densely innervate the infundibular stalk and the lamina terminalis, with KISS1-ir axon varicosities found in the ventral periventricular nucleus, preoptic nuclei, paraventricular nucleus, the infundibular nucleus, and the tuberal subdivision of the supraoptic nucleus. Scattered KISS1-ir fibers localize in the VMH and DMH and appear to innervate the medial septum and stria terminalis. Moreover, double-immunostaining for kisspeptin and GnRH reveals overlapping networks of GnRH- and immunoreactive kisspeptin-containing axons in circumventricular organs, including the ME, and contact between immunoreactive kisspeptin-contain-

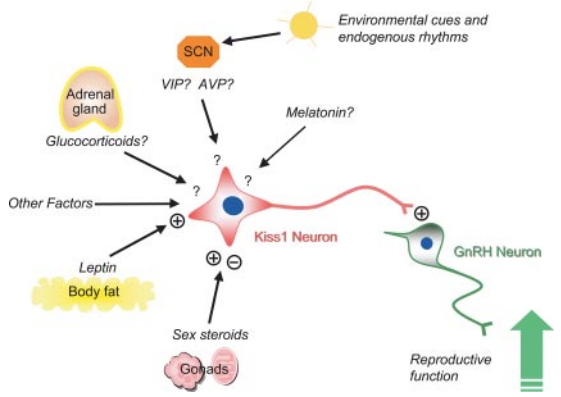

FIG. 10. Kisspeptin neurons may act as central processors for relaying signals from the periphery to GnRH neurons. Metabolic and environmental factors regulate reproductive function, which ensures that reproduction proceeds only when metabolic and environmental conditions are favorable. Kisspeptin stimulates GnRH secretion, and *Kiss1* mRNA is both negatively and positively regulated by sex steroids. The expression of Kiss1 may be induced by leptin, whose plasma levels reflect the state of metabolic reserves. Kisspeptin neurons may also receive input from the hypothalamic-pituitary-adrenal axis and from environmental cues such as time of day via the SCN of the hypothalamus and day length via melatonin from the pineal gland. AVP, Arginine vasopressin; VIP, vasoactive intestinal peptide. [Modified from Dungan *et al.*, 2006 (203)].

ing axon varicosities and GnRH neuronal dendrites (103). Collectively, the finding that KISS1 and KISS1R are expressed throughout the body suggest that kisspeptin plays a role not only in cell proliferation, metastasis and GnRH/gonadotropin secretion, but also in other physiological processes in various organ systems.

V. Molecular Physiology of Kiss1 Neurons

The molecular constitution of Kiss1 neurons is beginning to unfold, but much is yet to be discovered about their molecular and morphological features. Control of Kiss1 neurons emanates from a variety of sources, including steroid hormone feedback, metabolic signals, and photoperiodic cues (Fig. 10). Consistent with their role as mediators of steroid feedback, most Kiss1 neurons express α-estrogen receptors (ERα) (79, 82, 92, 104–106) (Fig. 11), ERβ (82), and progesterone receptors (PR) (106, 107) (Fig. 12). About 40% of Kiss1-expressing neurons in the ARC of the mouse express the mRNA for the active form of the leptin receptor (*Ob-Rb*) (108), thus providing a potential link between nutrition and reproduction. Numerous studies indicate that Kiss1 neurons are regulated by photoperiod (48, 52, 76, 77, 94, 107), but many questions remain unanswered with respect to the pathways (direct/indirect) and intermediates [*e.g.*, melatonin (109)] by which photoperiodic cues are relayed to kisspeptin neurons.

An important study by Goodman *et al.* (110) describes a subpopulation of ovine kisspeptin neurons in the ARC that coexpress dynorphin A and neurokinin B (NKB), and quite likely ERα and PR, because these steroid hormone receptors are expressed in nearly all dynorphin and NKB neurons in the ARC (111, 112) (Fig. 13). This is the first study to provide direct evidence that kisspeptin neurons contain additional neuropeptides involved in reproductive control. Other investigations provide implicit evidence for a similar coexpression phenomenon in other species, including the rat, mouse, and human. For example, there is extensive colocalization of NKB and dynorphin in the ARC of the rat (113). Because these neurons all express ERα (113), we can reasonably infer coexpression of kisspeptin, NKB, and dynorphin in the ARC of that species as well. Rometo *et al.* (102) observed similar distribution and morphology of NKB- and kisspeptin-containing neurons in the infundibular (ARC) nucleus of postmenopausal women, suggesting the expression of NKB and kisspeptin in the same cells. Moreover, approximately half of glutamatergic neurons in the ARC of sheep express ERα (114), indicating the presence of a discrete population of kisspeptin/dynorphin/NKB/glutamate estrogen-responsive neurons in the ARC.

Navarro and his colleagues (104, 115) have verified that kisspeptin, NKB, and dynorphin are all colocalized in cells of the ARC in the mouse, where all three of these neuropeptides are regulated by estradiol. Moreover, in this species kisspeptin neurons in the ARC also express the cognate receptors for NKB and dynorphin (*i.e.*, NK3 and κ-opiate receptor, respectively) (115), suggesting the existence of autosynaptic contacts among kisspeptin/dynorphin/NKB neurons in this region. Indeed,

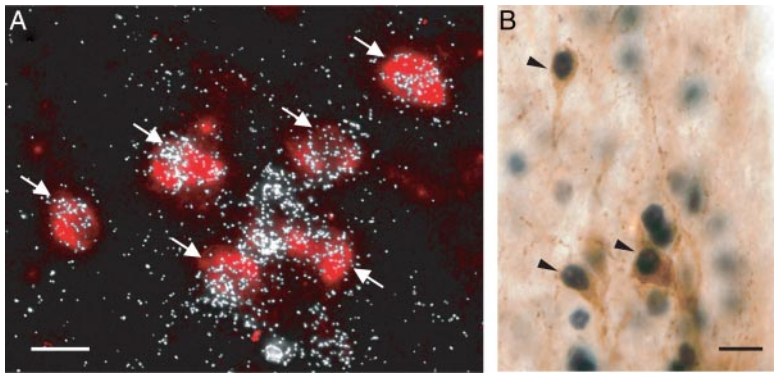

FIG. 11. Kisspeptin neurons express ERα. A, Representative photomicrograph showing coexpression of *Kiss1* mRNA with ERα in the AVPV of the female mouse. *Kiss1* mRNA-expressing cells were visualized with Vector Red substrate, and ERα was marked by the presence of clusters of silver grains (*white dots*). *Arrows* indicate *Kiss1* neurons that coexpress ERα. Scale bar, 20 μm. B, Dual-label immunocytochemistry showing kisspeptin neurons (*brown*) with ERα-immunoreactive nuclei (*black*) in the AVPV of the female mouse. *Arrowheads* indicate dual-labeled cells. Scale bar, 10 μm. [Modified with permission from Smith *et al.*, 2005 (104), © The Endocrine Society, and Clarkson *et al.*, 2008 (106)].

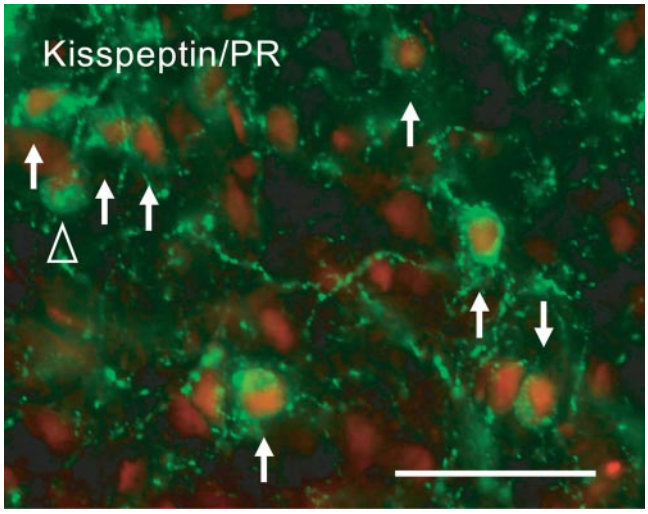

FIG. 12. Expression of PR in Kiss1 neurons. Photomicrograph of cells costaining for kisspeptin (*green*) and PR (*red*) in the ARC of the ewe brain. *Arrows* indicate cells containing both kisspeptin and PR. The *open arrowhead* indicates a kisspeptin-positive PR-negative cell. Scale bar, 50 μm. [Modified with permission from Smith *et al.*, 2007 (107) © The Endocrine Society].

a preliminary report from Inyushkin *et al.* (116) testifies to the appearance of Kiss1-ir synaptic contacts on Kiss1 neurons in the ARC—reinforcing the notion of an autosynaptically regulated network of kisspeptin neurons in this area. Furthermore, both symmetric and asymmetric synapses were observed (116), suggesting that Kiss1 neurons exert both stimulatory and inhibitory influences on themselves and one another. A recent study by Navarro *et al.* (115) demonstrates that NKB agonists inhibit LH secretion in ovariectomized mice; moreover, they demonstrate that mice bearing targeted deletions of either the *dynorphin* or *κ-opiate receptor* gene show a diminished postcastration rise in LH, implying that NKB and dynorphin signaling play key roles in the regulation of GnRH/LH secretion. Together, these observations suggest that kisspeptin, dynorphin, NKB (and perhaps glutamate) participate in the regulation of pulsatile GnRH secretion. Indeed, Navarro *et al.* (115) propose a model in which dynorphin and NKB act autosynaptically on kisspeptin neurons (directly and/or indirectly) to generate discrete pulses of kisspeptin, which in turn drives pulsatile GnRH and LH secretion. This idea is consistent with the observations of Keen *et al.* (89), showing apparent coincidence of pulsatile kisspeptin and GnRH secretion, as measured in the ME of the monkey. It is likely that other peptidergic systems and classical neurotransmitters, such as glutamate and γ-aminobutyric acid, also play important roles in the regulation of kisspeptin activity in the ARC—this subject is ripe for investigation (117).

In the AVPV, the story is different from the ARC. Kisspeptin neurons in the AVPV express neither dynorphin nor NKB in any species studied to date. However, in the mouse, kisspeptin neurons in the AVPV appear to coexpress tyrosine hydroxylase (118), suggesting that these cells may be dopaminergic—although this does not appear to be the case in the rat, where few, if any, kisspeptin neurons in the AVPV appear to express tyrosine hydroxylase (75). The differential expression of various cotransmitters with kisspeptin (ARC *vs.* AVPV) makes a compelling case that these two populations of "kisspeptin" neurons are phenotypically unique—not only in their molecular fingerprint, but also in their physiological function.

Little is known about the afferent inputs to Kiss1 neurons (besides possible autosynaptic processes). We can surmise that Kiss1 neurons in the AVPV of the rodent are likely to receive afferent input from the suprachiasmatic nucleus (SCN) because that region projects to the AVPV and is involved in the timing of the LH surge (119) (Fig. 10). The molecular identity of the neurotransmitters involved is currently unknown, but they could be arginine vasopressin or vasoactive intestinal peptide because these are the major efferent projections from the SCN (119, 120); however, this remains to be investigated.

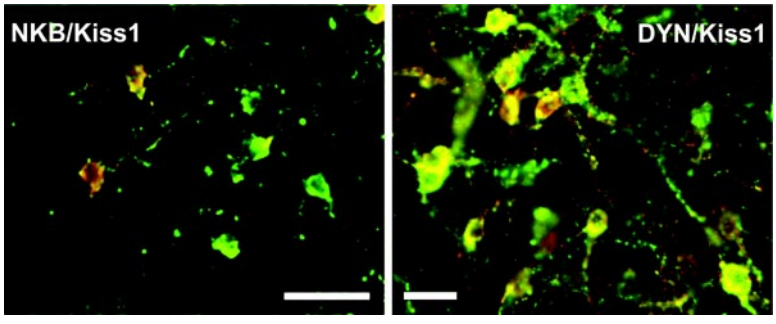

FIG. 13. Coexpression of NKB/Kiss1 and dynorphin (DYN)/Kiss1 in the middle ARC of the ewe. Overlay of *red* (Kiss1 neurons) and *green* (NKB) fluorescent images (left; scale bar, 50 μm) and overlay of *red* (DYN) and *green* (Kiss1 neurons) fluorescent images (right; scale bar, 20 μm), illustrating colocalization of kisspeptin with NKB and DYN with kisspeptin, respectively. Double-labeling appears *brown*. [Modified with permission from Goodman *et al.*, 2007 (110) © The Endocrine Society].

VI. Comparative Physiology

Animals employ various strategies to optimize reproductive success, including timing reproduction to the ideal season of birth (*e.g.*, fall breeding for sheep and spring breeding for hamsters) and timing the event of ovulation to occur within a narrow window to maximize the opportunity of successful mating (*e.g.*, just before the onset of darkness and activity in the rat and nonphotoperiodic mechanisms in the primate). These different strategies are reflected in unique and diverse organization of Kiss1 cir-

cuitry in the brain across species. Some aspects of kisspeptin anatomy and physiology are highly conserved across species, such as the stimulatory effect of kisspeptin on GnRH and the inhibitory action of estradiol in *Kiss1* gene expression in the MBH. However, other aspects of Kiss1 anatomy and physiology are unique to particular species—such as the mechanisms that govern the preovulatory surge of GnRH/LH. We will try to identify those aspects of kisspeptin signaling in the brain that are widely shared among species and those that are unique to certain animal groups.

A. Direct and indirect effects of kisspeptin on GnRH neurons

Several major lines of evidence suggest that kisspeptin signals directly to GnRH neurons (121). First, the majority of GnRH neurons express *Kiss1r* (46, 70, 71, 80). Second, kisspeptin-ir fibers are found in close association with GnRH neurons (42, 73, 94, 96). Third, kisspeptin can act directly to depolarize and increase firing rates of GnRH neurons *in vitro* (17, 19, 70, 122–125). It should be noted that although kisspeptin may act through traditional synaptic mechanisms to stimulate GnRH secretion, it may also act directly in a nonsynaptic manner, particularly in the ME (91, 92, 96, 99, 126). In addition to acting directly on GnRH neurons, there is growing evidence to suggest that kisspeptin also acts on intermediary neurons, such as GABAergic cells, to regulate GnRH secretion (123, 127).

B. Pituitary effects

When kisspeptin acts at the level of the hypothalamus to increase GnRH secretion, it produces an increase in LH release from the pituitary. However, some studies suggest that kisspeptin may also act at the level of the pituitary to evoke LH secretion through a direct action on the gonadotropes. A detailed description of the action of kisspeptin on the pituitary has recently been reviewed (128). Indeed, it would appear that both Kiss1 and Kiss1r are expressed in the pituitary, specifically in gonadotropes, and are differentially regulated by sex steroids (42, 86, 95, 129). The presence of a functional kisspeptin receptor in the pituitary, combined with the finding that kisspeptin is released in ovine hypophyseal portal blood, suggests kisspeptin action at the level of the pituitary to modulate gonadotropin secretion (95). This proposition is supported by *in vitro* studies demonstrating a stimulatory increase in gonadotropin secretion from pituitary fragments or cells treated with kisspeptin (129–131). Taken together, these findings would argue for a dual action of kisspeptin, at the pituitary and hypothalamus, with the collective outcome of increased gonadotropin secretion.

However, there is controversy about this conclusion, and it remains unclear whether kisspeptin acts as a true hypophysiotropic factor (*i.e.*, released from the brain and transported via the portal circulation to act on gonadotropes) to regulate LH secretion. Although kisspeptin can be measured in hypophyseal portal blood, levels are similarly low in both ovariectomized ewes and ewes treated with estrogen to induce an LH surge (95), suggesting that the action of kisspeptin at the pituitary does not greatly affect the release of LH. Utilizing the hypothalamo-pituitary disconnection sheep model, Smith *et al.* (95) examined the *in vivo* relevance of kisspeptin at the level of the pituitary. In this model, kisspeptin treatment had no effect on LH secretion (during steady-state conditions and the estrogen-induced surge) (95), indicating that any effect of kisspeptin on LH secretion occurs upstream of the pituitary. Furthermore, a GnRH antagonist blocks the kisspeptin-induced increase in LH, again suggestive of (but not proving) a supra-pituitary action of kisspeptin (72, 80) (Fig. 14). Thus, whereas some evidence supports a role of kisspeptin action at the pituitary, other findings would suggest otherwise; further experiments are necessary to identify the role of kisspeptin at the pituitary.

C. Continuous *vs.* pulsatile exposure to kisspeptin

After an initial stimulation, a continuous (chronic) exposure of the pituitary to GnRH (or agonists) eventually causes suppression of gonadotropin secretion (132) through down-regulation and sensitization of the GnRH receptors (133–136). Because kisspeptin is known to activate the brain-gonadotrope axis, several groups have examined whether a constant infusion of kisspeptin would produce the same inhibitory effect on gonadotropin secretion as GnRH. Indeed, continuous delivery of exogenous kisspeptin appears to desensitize Kiss1r, resulting in decreased LH secretion in agonadal juvenile and adult male monkeys and testicular degeneration in adult male rats (137–139). In contrast, repeated peripheral injections of kisspeptin elicit unrestrained LH pulses in male rats and monkeys (140, 141), implying that the efficacy of kisspep-

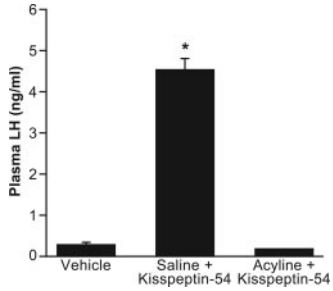

FIG. 14. The GnRH antagonist, acyline, blocks the effects of kisspeptin-54 on plasma LH in the mouse. Kisspeptin-54 (50 pmol) and vehicle were administered into the lateral ventricle. Pretreatment with the GnRH antagonist, acyline (50 μg) or saline was given sc. *, $P < 0.001$ saline + kisspeptin-54 *vs.* all other treatments. [Modified with permission from Gottsch *et al.*, 2004 (72) © The Endocrine Society].

tin to drive LH secretion depends on its pulsatile nature, much like for GnRH. Interestingly, sustained (30 or 48 h) iv kisspeptin treatment was effective in seasonally acyclic ewes (anestrous season), resulting in ovulation in 80% of animals (compared with 20% controls) (142). It is not clear whether this finding reflects a difference in the way sheep respond to continuous exposure to kisspeptin compared with other species or is due to differences between studies in the dose and mode of kisspeptin administration. Because desensitization could have a major impact on the efficacy of kisspeptin analogs and antagonists when used as contraceptives or to treat reproductive disorders, a better understanding of this phenomenon is needed.

D. Negative feedback action of sex steroids on *Kiss1* gene expression in ARC

The ability of testosterone to act at the level of the hypothalamus to suppress GnRH and thereby regulate gonadotropin secretion and testicular function in the male is a classic example of negative feedback. Because GnRH neurons appear to lack both the androgen receptor (AR) and ERα (in either sex), some intermediary neuronal system is thought to indirectly relay the feedback signal from the gonad to GnRH neurons. Recent observations suggest that kisspeptin neurons may represent an important component of this negative feedback loop (Fig. 15). After castration in mice, rats, hamsters, and monkeys, levels of *Kiss1* mRNA increase dramatically in the mediobasal hypothalamus, specifically the ARC; moreover, this effect can be reversed with sex steroid replacement (*e.g.*, with testosterone, estradiol, or dihydrotestosterone) (76, 80, 100, 105, 143). A castration-induced increase in *Kiss1* expression in the ARC coincides with the increase in GnRH and gonadotropin secretion that results after removal of the negative feedback action of testosterone (*i.e.*, after castration) (105). The postcastration rise in LH can be blocked by kisspeptin antagonists in the male rat and mouse (43). Kisspeptin neurons in the ARC of the mouse appear to express the AR and ERα (105), reflecting that they are direct targets for the action of sex steroids; moreover, studies of male mice with null mutations in the ER (ERαKOs) and hypomorphic alleles of the AR implicate both ERα- and AR-dependent regulation of *Kiss1* gene expression in the ARC (105). Taken together, these observations provide convincing evidence that Kiss1/Kiss1r signaling (in the ARC) mediates the negative feedback regulation of GnRH/LH secretion—at least in the male.

In the female mammal, during most days of the estrous (and menstrual) cycle, the negative feedback control of gonadotropin secretion predominates, and a relatively low plasma level of gonadal steroids restrains GnRH and LH secretion. Kisspeptin neurons appear to play a key role in the negative feedback action of estradiol in the female (Fig. 15), as they do for testosterone in the male. The expression of *Kiss1* mRNA in the ARC changes as a function of the estrous cycle in the rat, with levels reaching nadirs at or around the time when estradiol levels are highest (82) (Fig. 16). Ovariectomy causes an increase in hypothalamic expression of *Kiss1* mRNA in the ARC of rodents, sheep, and monkeys (94, 102, 104, 107, 143–145). The increase in expression of *Kiss1* is reversible upon treatment with

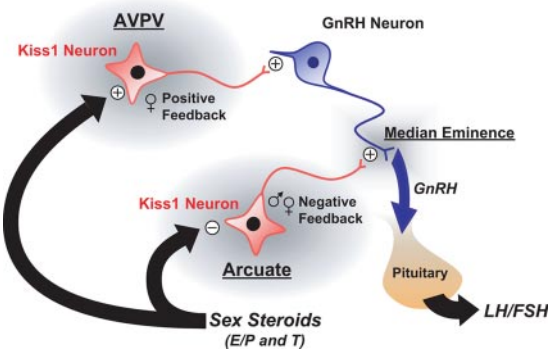

FIG. 15. A schematic representation of our current understanding of Kiss1 signaling in the forebrain of the mouse. Kisspeptin stimulates GnRH secretion by a direct effect on GnRH neurons, most of which express the kisspeptin receptor, Kiss1r. Neurons that express *Kiss1* mRNA reside in the AVPV and the ARC (arcuate). Kiss1 neurons in the ARC appear to be involved in the negative feedback regulation of GnRH/LH by sex steroids. The expression of *Kiss1* mRNA in the arcuate is inhibited by estradiol (E), progesterone (P), and testosterone (T). These same hormones induce *Kiss1* mRNA expression in the AVPV, where Kiss1 neurons are thought to be involved in the positive feedback regulation of GnRH/LH. [Modified with permission from Gottsch *et al.*, 2006 (204) © Elsevier].

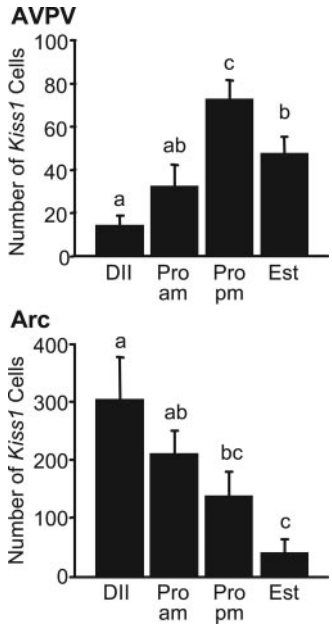

FIG. 16. *Kiss1* expression in the AVPV and ARC over the estrous cycle of the rat. Values without common notations (a, b, c) differ significantly ($P < 0.05$). Values are the mean ± SEM. DII, Diestrus; Pro am, proestrus 2 h after lights on; Pro pm, proestrus 1 h before lights off; Est, estrus. [From Smith *et al.*, 2006 (82)].

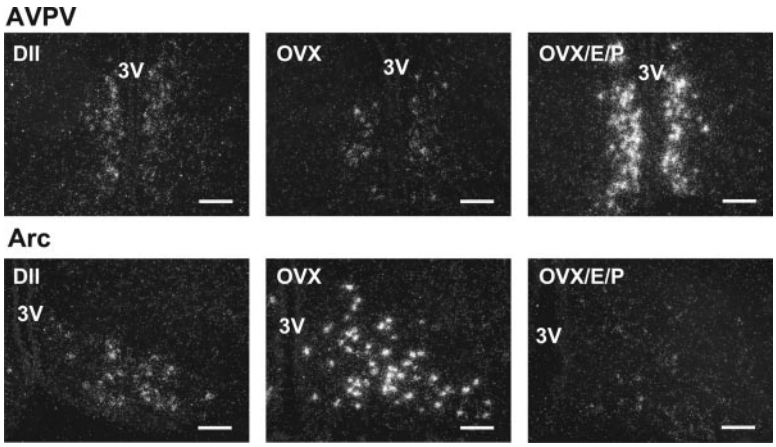

FIG. 17. Dark-field photomicrographs showing *Kiss1* mRNA-expressing cells (as reflected by the presence of white clusters of silver grains) in representative sections of the AVPV and ARC from diestrus (DII), ovariectomized (OVX) and ovariectomized/estradiol/progesterone (OVX/E/P)-treated female rats. 3V, Third ventricle. Scale bars, 100 μm. [From Smith *et al.*, 2006 (82)].

estradiol (82, 94, 102, 104, 107, 143, 144) (Fig. 17). Finally, female mice bearing targeted deletions of *Kiss1r* do not show a postcastration rise in LH— despite exhibiting a dramatic increase in the expression of *Kiss1* mRNA (144). These observations suggest that kisspeptin neurons in the ARC of both the male and female provide tonic drive to GnRH neuronal activity, which is modulated by the negative feedback effects of gonadal steroids (testosterone in the male and estradiol in the female). However, the situation with kisspeptin neurons in the AVPV (of the rodent) is different.

E. Circadian signals and positive feedback action of estradiol on *Kiss1* gene expression in AVPV

Early on proestrus in rodents (or late in the follicular phase of the menstrual cycle in primates), the rising tide of estradiol in the plasma triggers a surge of GnRH and LH secretion, which induces ovulation. In rodents, this so-called positive feedback effect of estradiol appears to involve estradiol-sensitive neurons in the AVPV, which act directly on GnRH neurons to stimulate the preovulatory surge of GnRH and thus LH (146, 147). Nearly all kisspeptin cells in the AVPV of the female rodent express ERα (104); moreover, the AVPV is a sexually dimorphic nucleus, with sexually differentiated expression of tyrosine hydroxylase (148), *Kiss1* (75), neurotensin (149), and other genes.

Kisspeptin neurons in the AVPV of rodents appear to play a central role in relaying the positive feedback effects of estradiol to GnRH neurons. First, treatment with a kisspeptin antiserum to block kisspeptin signaling completely abolishes the LH surge in female rats (42, 79) (Fig. 3). Second, in the mouse, the expression of *Kiss1* mRNA in the AVPV is dramatically induced by estradiol (104, 144). Third, in the rat, the expression of *Kiss1* mRNA in the AVPV peaks at a time coincident with the GnRH/LH surge, and Kiss1 neurons in the AVPV show Fos induction at precisely this time (79, 82) (Fig. 18). Fourth, a population of rodent ERα-positive neurons makes direct synaptic contact with GnRH neurons (146, 147), and these neurons are likely to be Kiss1 neurons (104). Finally, a report by Clarkson *et al.* (106) showed that whereas normal wild-type mice that have been ovariectomized and treated with both estradiol and progesterone show a clear LH surge, mice bearing targeted deletions in *Kiss1r* appear to lack this capacity. Moreover, in this same study, approximately 50% of GnRH neurons in wild-type mice showed Fos expression coincident with the LH surge, whereas none of the mutant animals showed evidence of Fos expression at this same time (106). Together, these observations imply that activation of kisspeptin neurons and their signaling to GnRH neurons is a prerequisite for generating the estradiol/progesterone-induced GnRH/LH surge in the female mouse.

Despite the compelling argument that kisspeptin signaling is inextricably linked to the preovulatory surge of GnRH and LH, there are two lines of evidence that add caution to this conclusion. First, a study by Dungan *et al.* (144) revealed that in another, independently produced line of *Kiss1r*-knockout (*GPR54* knockout) mice, ovariectomized knockout females treated with estradiol (alone) retain the capacity to elicit a GnRH/LH surge and Fos induction in GnRH neurons—virtually identical to wild-type controls, indicating kisspeptin signaling in these mice with this paradigm is not an absolute prerequisite for induction of the GnRH/LH surge. Although Clarkson *et al.* (106) and Dungan *et al.* (144) have provided evidence for having produced a complete knockout of the *Kiss1r* gene, the method these groups used to generate a GnRH/LH surge in ovariectomized animals differed, which might explain their different results (and conclusions). The protocol used to produce a surge in the Dungan *et al.* (144) study involved sustained treatment with estradiol alone (which produces a diurnal GnRH/LH surge that persists for many days), whereas that used by Clarkson *et al.* (106) involved a combination of estradiol and progesterone to generate a single (nonreplicating) surge (which produces a single GnRH/LH surge, instead of a daily event). In any case, it is conceivable that these two methods activate different pathways to produce the GnRH/LH surge, which could involve a differential dependency on kisspeptin signaling. It is interesting to note that the estradiol alone treatment paradigm generates a daily surge of *Kiss1* gene expression and *c-fos* expression in kisspeptin neurons in the AVPV, which is accompanied by an LH surge even in constant darkness— presumably reflecting circadian activation by the suprachiasmatic nucleus (150).

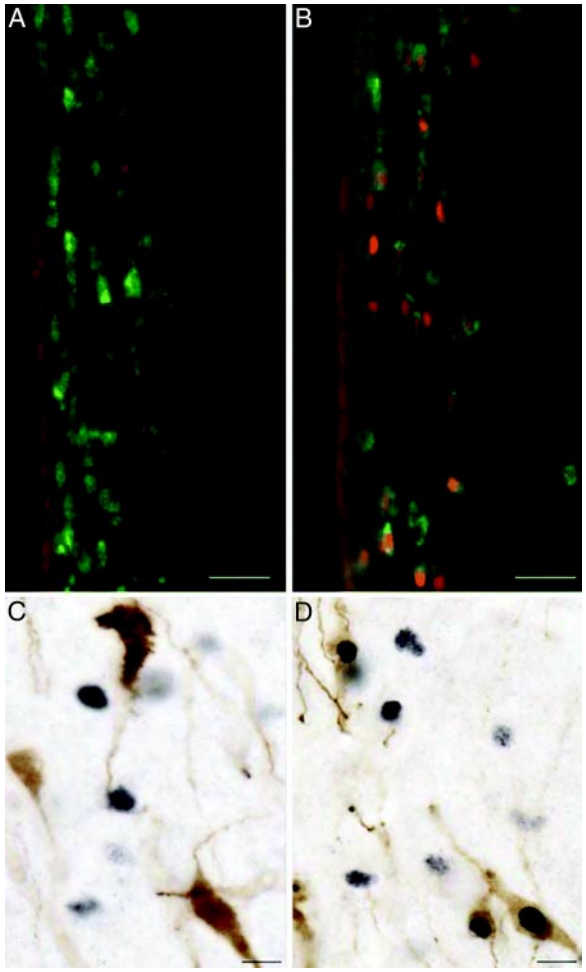

FIG. 18. Fos expression in the AVPV of the rat before and during an LH surge. A and B, Photomicrographs showing Kiss1 neurons (*green*) in the AVPV on diestrus II (A) and proestrus (B), with the induction of Fos (*red*) in Kiss1 neurons on the afternoon of proestrus (compare A and B). Scale bars, 25 μm. C and D, Photomicrographs showing GnRH neurons (*brown*) and Fos (*black*) on diestrus II (C) and their coexpression on the afternoon of proestrus (D). Scale bars, 10 μm. [From Smith *et al.*, 2006 (82)].

A second set of observations from Seminara and her colleagues also argues that kisspeptin signaling is not an absolute prerequisite to sustain some degree of activity of the brain-gonadotropin axis. Their studies show that a subset of animals in another independently derived line of *Kiss1r* knockout mice and a separate line of *Kiss1* knockout mice retain the capacity to show some degree of gonadotropin secretion and ovarian cyclicity (based on vaginal smears)—albeit lacking regularity and evidence of ovulation (68, 151). Moreover, patients with various mutations in *KISS1R* show variable reproductive phenotypes (even those with an identical mutation), which in some cases indicates a modest level of gonadotropin secretion (low levels of pulsatile LH secretion) and gonadal activity (8, 152, 153), although we cannot discount the possibility that these mutations do not completely disable the kisspeptin receptor and thus kisspeptin signaling to GnRH neurons. Together, these observations would suggest that either some of the various mutations are not fully disabling to the kisspeptin receptor and signaling pathway or that GnRH secretion can occur at low levels independently of trophic activation by kisspeptin. This could cause some degree of GnRH/gonadotropin-dependent reproductive activity to persist in many animal models in which kisspeptin signaling would appear to be inactivated (or severely compromised), whereas in other models, inactivation leads to complete reproductive failure.

How might the brain-gonadotropin axis retain some activity even when kisspeptin signaling has been completely disabled? First, there may be a compensatory process that occurs during development, which drives GnRH secretion and affects a partial rescue of the reproductive phenotype. Second, there may be redundancy in the circuits that drive the GnRH/LH surge, which could partially rescue the phenotype, such as the neurotensin pathway in the AVPV (154). Third, the activity of one of the kisspeptin cotransmitters could sustain some level of GnRH activity (*e.g.*, perhaps glutamate or dopamine produced by Kiss1 cells in the AVPV). These ideas remain untested.

Although a strong case can be made that the negative feedback effect of estradiol and testosterone on GnRH secretion is mediated by kisspeptin/dynorphin/NKB-producing neurons in the ARC of the mammals studied to date, the same unifying principle does not apply in the case of positive feedback. In the rodent, the ability of estradiol to evoke a GnRH/LH surge would appear to be mediated by kisspeptin neurons in the AVPV. However, in the ewe and primates, there is no homolog of the AVPV as found in the rodent. In the ewe, the positive feedback effects of estradiol appear to be mediated by kisspeptin neurons in the rostral region of the mediobasal hypothalamus, as evidenced by the up-regulation of *Kiss1* mRNA in the rostral ARC during the preovulatory period (155); however, we lack a fundamental understanding of how (in the ewe) kisspeptin neurons in the ARC mediate both negative and positive feedback. As we learn more about the molecular fingerprint of Kiss1 neurons in the ARC of the sheep, it may turn out that there are several "phenotypes" of Kiss1 neurons comprised within the MBH— one involved in negative feedback, and another involved in positive feedback. At the moment, this is a matter of pure conjecture. In the case of primate species (*e.g.*, monkey and human), we know that the neuroendocrine mechanisms that generate the preovulatory GnRH/LH surge are also different from the rodent (and the ewe, for that matter; see Ref. 156). Unfortunately, we currently have no insight into the role of kisspeptin in generating the GnRH/LH surge in any primate species. Taken together, these observations suggest site- and species-specific roles for kisspeptin neurons in mediating steroid hormone signaling

to GnRH neurons—and should serve as an open invitation for further investigation.

F. Differential regulation of *Kiss1* gene expression by estradiol in the brain

The molecular mechanism by which estradiol differentially regulates *Kiss1* in the ARC and AVPV is unknown. ERα can exert a multiplicity of cellular effects, depending upon its interactions with various signaling pathways. The "classic" pathway involves estradiol binding to estrogen response elements (EREs) in the gene promoter to alter transcription (ERE-dependent). An alternative ERα signaling pathway involves ERE-independent (nonclassical) mechanisms, which include protein-protein interactions at heterologous response elements, such as SP-1 and AP-1 sites. Recently, a line of mice has been developed that permits distinguishing between ERE-dependent and -independent signaling pathways *in vivo* (157). In these nonclassical ERα knock-in mice, a single allele of a mutated ERα ($ER\alpha^{AA}$) confers nonclassical ERα signaling in the absence of classical signaling ($ER\alpha^{AA/-}$). Analysis of these mice indicates that ERE-independent signaling is sufficient for negative feedback regulation of LH, whereas positive feedback requires ERE-mediated transcriptional regulation of target genes. Recent preliminary studies comparing levels of *Kiss1* mRNA in $ER\alpha^{+/+}$, $ER\alpha^{-/-}$, and $ER\alpha^{AA/-}$ adult female mice demonstrate that the effect of estradiol on the expression of *Kiss1* is mediated by classical ERE pathways in the AVPV and by nonclassical pathways in the ARC (158). Thus, the same mechanisms that mediate the positive and negative feedback effects of estradiol on gonadotropin secretion also appear to mediate the effects of estradiol on *Kiss1* expression in the murine AVPV and ARC, respectively. This is consistent with the argument that kisspeptin neurons in the ARC and AVPV of the rodent participate in the negative and positive feedback regulation of gonadotropin secretion. Furthermore, this same study found that estradiol inhibits dynorphin gene expression in the ARC through a classical ERα pathway (158). Because *Kiss1* and dynorphin are expressed in the same population of neurons in the ARC, these results demonstrate that the estradiol-dependent regulation of coexpressed genes in single neurons can occur by different ERα signaling mechanisms.

G. Kisspeptin in pregnancy, lactation, and aging

In addition to the menstrual (or estrous) cycle in which kisspeptin may participate, an adult female can experience a variety of functional reproductive states. In pregnancy, for example, rats maintain LH and FSH secretory responses to kisspeptin and exhibit an increase in hypothalamic *Kiss1* gene expression (159). Moreover, a dramatic increase in circulating levels of kisspeptin occurs during pregnancy in humans (160). This circulating kisspeptin is derived mainly from the placenta, which expresses both *Kiss1* and *Kiss1r* (1, 3, 4). Concentrated in the syncytiotrophoblasts, kisspeptin may be involved in the regulation of trophoblast invasion during the first trimester, when plasma kisspeptin is elevated (160). The finding that kisspeptin suppresses the motility, invasion, and growth of Kiss1r-transfected CHO cells *in vitro* (161) further supports a role of kisspeptin in trophoblast invasion. But, high levels of circulating plasma kisspeptin suggest kisspeptin may have other roles during pregnancy and raises a number of questions. Might continuous high levels of plasma kisspeptin "down-regulate" the hypothalamic-pituitary gonadal axis and be responsible in part for the cessation of reproductive cycles in the female? Could "abnormal" levels of kisspeptin somehow contribute to problems associated with pregnancy, such as gestational diabetes, preeclampsia, or preterm labor? Clearly, there are many areas to explore regarding the role(s) of kisspeptin in pregnancy.

Despite maintaining their LH and FSH responsiveness to kisspeptin (159), lactating rats have reduced expression of *Kiss1* mRNA in the ARC region and *Kiss1r* mRNA expression in the AVPV (162), providing a possible mechanism to explain the reduction of LH secretion during lactation. The suckling stimulus appears to be responsible for the suppression of *Kiss1* mRNA expression in the ARC (162), and it is interesting to note that neural inputs derived from the suckling stimulus activate neurons projecting to the ARC (163). Because lactation is associated with low levels of estradiol (which would ordinarily increase *Kiss1* expression in the ARC), it is remarkable that the expression of *Kiss1* mRNA in the ARC is so low during lactation (162). What is the physiological significance of having suppressed levels of *Kiss1* in the ARC? Perhaps suckling-induced reduction in kisspeptin and Kiss1r represents a mechanism whereby estrous and menstrual cycles are shut down during lactation (lactational amenorrhea).

Aging takes a toll on the reproductive system—most obviously in females (164). This becomes reflected in disrupted cycles and eventually constant estrus (or diestrus) in rodents and menopause in primates. This does not appear to be due to a defect in the ARC, but a serious defect in the AVPV, because preliminary data indicate that declining expression of kisspeptin in the AVPV occurs in middle-aged female rats, reducing the stimulatory drive of kisspeptin to GnRH neurons and delaying the estradiol-mediated GnRH/LH surge (165). Of interest, menopause in women is associated with hypertrophy of Kiss1 neurons and increased expression of *Kiss1* mRNA in the MBH (infundibular nucleus), perhaps reflecting reduced circulating levels of estradiol and the reduction of negative feedback (102, 145). Although kisspeptin appears to be a rel-

evant signaling hormone in reproductive aging, its precise role is yet to be defined.

H. Metabolic regulation

Evidence suggests that the activity of Kiss1 neurons is influenced by body weight, nutrition, metabolism, and hormonal signals (166, 167, 205). As noted previously, a significant fraction of Kiss1 neurons in the ARC express the leptin receptor, Ob-Rb (108). Moreover, *Kiss1* mRNA is significantly reduced in obese *ob/ob* mice compared with wild-type controls (108). In other experimental models in which the leptin receptor is dysfunctional as a result of a mutation, such as the obese, diabetic Zucker rat (*fa/fa*), reproduction is also impaired, but treatment with exogenous kisspeptin can induce an acute release of LH in this model, suggesting that the kisspeptin signaling might be responsible for their dysfunction (168). In rats with streptozotocin-induced diabetes, hypothalamic levels of *Kiss1* mRNA are decreased, which is accompanied by reduced circulating levels of gonadotropins; however, the hypogonadotropic state associated with streptozotocin-induced diabetes can be rescued by kisspeptin administration, implying that reduced kisspeptin signaling may explain the reproductive failure that often accompanies diabetes (169, 170). In states of undernutrition (or fasting), which reduce gonadotropin secretion as well as the expression of *Kiss1*, exogenous kisspeptin administration can reinstate reproductive function (168, 171, 172). However, it is conceivable that the apparent rescue of the reproductive axis associated with poor nutrition or diabetes that occurs with kisspeptin simply reflects its ability to activate GnRH neurons downstream of the mechanisms that are impaired in these altered metabolic states. Collectively, these findings point to a potentially important role of Kiss1 neurons in regulation of reproduction by metabolic factors.

I. Seasonality

The role of kisspeptin in the photoperiodic control of reproduction has been examined in several recent reviews (173–175). Seasonal breeders, such as hamsters and sheep, restrict fertility to a particular time of year to ensure the birth of offspring during favorable environmental conditions. Photoperiod is a predominant environmental cue that governs the pattern of melatonin secretion from the pineal gland, which helps the animal determine season. For example, reproductive activity of the Syrian hamster is promoted by long summer days and inhibited by short winter days. Levels of *Kiss1* mRNA in the ARC are reduced in male Syrian hamsters after transfer from long-day to short-day conditions, which leads to reproductive quiescence (76). This seasonal change appears to be melatonin-dependent because pineal gland ablation prevents this short-day induced down-regulation of *Kiss1* expression (76); however, it is unclear whether melatonin acts directly on Kiss1 neurons. Remarkably, chronic infusion of kisspeptin restores testicular activity in Syrian hamsters despite persisting photoinhibitory conditions (76). Both male and female Siberian hamsters held in short-day conditions exhibit a reduced response to exogenous kisspeptin treatment and show negligible kisspeptin expression in the AVPV and high expression in the ARC (77). In long-day conditions, however, this expression is reversed, with marked kisspeptin staining in the AVPV and only minor expression in the ARC (77, 78). It should be noted that some studies performed in the hamster have been confounded by a lack of specificity (and proper validation) of the antibodies used to detect kisspeptin by immunocytochemistry. Furthermore, interpreting the results of semiquantitative immunocytochemistry can be challenging. For example, when there is little apparent expression of kisspeptin, this could mean either that little kisspeptin is being made (thus none appears) or that whatever is being made (perhaps even in great abundance) is rapidly released. Thus, analysis of staining intensity by immunocytochemistry should be interpreted with caution. Nevertheless, it does appear that low levels of kisspeptin and a reduced sensitivity to the hormone may contribute to the reproductive quiescence induced by short-day photoperiods. Investigations in the hamster are complicated by the fact that kisspeptin activity in the two species (Syrian and Siberian) appears to respond differently to short days, which makes generalizations difficult.

The sheep, another seasonally breeding species, becomes reproductively active as the days become shorter in autumn and becomes quiescent as the days become longer. The expression of kisspeptin also varies with season in the sheep. For example, Kiss1 expression is lower and there are fewer kisspeptin terminal contacts onto GnRH neurons during the nonbreeding period (long days) compared with the breeding period (short days) (94, 107). Moreover, during anestrus (non breeding) season, infusion of kisspeptin for several days can induce ovulation (142). Hence, there appears to be a fundamental contribution made by kisspeptin signaling to regulate seasonal breeding in a variety of species.

J. Puberty

Several recent reviews have focused on the role of kisspeptin in puberty (176–182). Humans and mice lacking a functional kisspeptin receptor do not progress normally to achieve puberty (7, 8). Many species exhibit a marked increase in Kiss1 and/or Kiss1r expression in association with the onset of puberty, suggesting that kisspeptin acts as gatekeeper for puberty (48, 51, 70, 90, 101, 168, 183).

In the mouse, the distribution of Kiss1 neurons and expression of *Kiss1* mRNA changes over development.

Clarkson *et al.* (183) have reported that the number of Kiss1 neurons in the AVPV/PeN increases exponentially from postnatal day 10 through puberty. In addition, using dual immunofluorescence, Clarkson and Herbison (73) have found that the proximity of appositions between kisspeptin fibers and GnRH neuronal somata increases at the time of puberty (Fig. 6), suggesting increased kisspeptin input to GnRH neurons at this developmental juncture. This increased input does not appear to be associated with an increase in the expression of kisspeptin receptors in GnRH neurons because Han *et al.* (70) have reported that the per cell content of *Kiss1r* mRNA does not differ between juvenile and adult animals. On the other hand, *Kiss1* expression in the AVPV is higher in the adult compared with juvenile mouse; moreover, the percentage of GnRH neurons responding to kisspeptin increases from approximately 25% in juveniles to approximately 45% in prepubertal mice to more than 90% in adults (70), suggesting that GnRH neurons become more sensitive to kisspeptin throughout postnatal development—without altering *Kiss1r* gene expression. Furthermore, central administration of submaximal doses of kisspeptin stimulates LH secretion in adult, but not prepubertal male mice (70). Central and peripheral administration of kisspeptin to juvenile female rats also stimulates LH release and ovulation (184) and advances the timing of vaginal opening (168).

Clarkson and Herbison (183) have proposed a model to explain the pubertal activation of gonadotropin secretion—based on the observation that estradiol increases the number of identifiable Kiss1 cells in the AVPV/PeN of prepubertal animals (as detected by immunocytochemistry). According to their model, estradiol in the prepubertal period stimulates Kiss1 neurons in the AVPV/PeN that activate GnRH neurons. Increased GnRH secretion then stimulates gonadotropin release, which subsequently drives further estradiol production from the ovary—thus producing a feed-forward activational loop. This putative mechanism would cause AVPV/PeN neurons to act as "estradiol-dependent 'amplifiers' of GnRH neuron activity." In support of this hypothesis, these investigators demonstrate that Kiss1 neurons are virtually absent in estradiol-deficient, aromatase knockout (ARKO) mice—which is perhaps not surprising because the expression of *Kiss1* in the AVPV/PeN has been shown to be estrogen/ERα/ERE-dependent (104, 158).

However, several other studies argue against the proposition that Kiss1 neurons in the AVPV/PeN play an inductive role in pubertal maturation (in either sex). First, adult female ARKO mice have been shown to have fully developed ovaries, containing numerous follicles, which never undergo ovulation—presumably reflecting a lack of estradiol-induced positive feedback (185). However, the very presence of developed follicles in these ARKO mice suggests that the gonadotropin drive associated with pubertal development is not dependent on the presence of Kiss1 neurons in the AVPV/PeN—because those neurons are absent in ARKO mice. Moreover, it is not apparent how the regenerative feedback system proposed by Clarkson and Herbison (183) would explain puberty in males, which have only a few scattered Kiss1 neurons in their AVPV/PeN, even in adulthood (73, 75). Thus, any possible role for *Kiss1* neurons of the AVPV/PeN in pubertal development is unresolved and remains a matter of lively debate.

To determine whether kisspeptin is involved in the regulation of puberty in primates, Shahab *et al.* (101) used real-time PCR to identify changes in the hypothalamic expression of *Kiss1* and *Kiss1r* around the time of puberty. In agonadal male monkeys, hypothalamic expression of *Kiss1* was greater in animals at the presumptive time of puberty than juvenile animals, whereas there was no difference in *Kiss1r* expression (101). In ovary-intact females, *Kiss1* transcript expression was 3-fold greater in midpubertal monkeys compared with juvenile or early pubertal animals, corresponding with a progressive increase in *Kiss1r* mRNA from juvenile to midpubertal stages (101). These observations suggest that increased kisspeptin signaling in the primate hypothalamus is responsible for initiating the transition from a hypogonadotropic state associated with a juvenile stage of development to a resurgence of pulsatile GnRH release at the time of puberty. Confirming this hypothesis is recent evidence from Keen *et al.* (89) in the Terasawa lab showing that an increase in kisspeptin-54 output, specifically an increase in kisspeptin-54 pulse frequency, occurs at the onset of puberty (as demonstrated via *in vivo* microdialysis in prepubertal and pubertal ovarian-intact female rhesus monkeys) (Fig. 19).

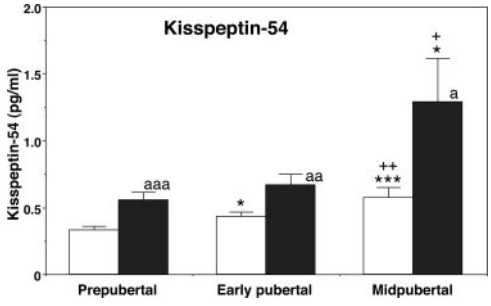

FIG. 19. Developmental changes in the release of kisspeptin-54 in push-pull perfusates in monkeys. Kisspeptin-54 levels gradually increase along with the pubertal increase in GnRH release. A nocturnal increase in kisspeptin-54 release is already observed in prepubertal monkeys and continues through the pubertal period. The number of animals at the prepubertal, early pubertal, and midpubertal stages was six, six, and five, respectively. *White bars*, Morning values; *black bars*, evening values. *, $P < 0.05$ *vs.* prepubertal; ***, $P < 0.001$ *vs.* prepubertal; +, $P < 0.05$ *vs.* early pubertal; ++, $P < 0.01$ *vs.* early pubertal; a, $P < 0.05$ *vs.* morning; aa, $P < 0.01$ *vs.* morning; aaa, $P < 0.001$ *vs.* morning. [Modified with permission from Keen *et al.*, 2008 (89) © The Endocrine Society].

Collectively, these findings provide strong support for an important role of kisspeptin—most likely produced from Kiss1 neurons in ARC—in initiating puberty, in many, if not all mammals. The next challenge is to figure out what triggers amplification of kisspeptin signaling at the time of puberty. Finally, despite our yearning to identify unified themes, we must remember that the molecular and cellular mechanisms that control the onset of puberty differ remarkably across Orders (*e.g.*, Rodentia, including mice and rats; Artiodactyla, including sheep; and Primates, including humans), and hence, the role of kisspeptin in gating pubertal maturation is likely to be different among these groups.

K. Sexual differentiation

Sexual differentiation of the Kiss1 circuits is species specific. For example, in the adult sheep (unlike the rat), the ARC is sexually differentiated, with ewes expressing higher numbers of Kiss1 neurons than rams. This finding is not surprising, given the putative role of the ovine ARC in mediating the sexually dimorphic GnRH/LH surge (155, 186). In the rodent, the AVPV is sexually differentiated, being larger and comprising more cells in the female than the male, reflecting sexual differentiation of many neuronal phenotypes, including tyrosine hydroxylase-positive neurons, neurotensin neurons, as well as Kiss1 neurons (73, 75, 79, 148, 149). Because the AVPV is thought to play a critical role in relaying the positive feedback effects of estradiol to GnRH neurons (82, 144), it is not surprising that the male rodent is incapable of generating a GnRH/LH surge. The sex difference in Kiss1 expression of the adult rodent is organized perinatally, as evidenced by the fact that neonatally androgenized females display a male-like pattern of *Kiss1* expression in the AVPV in adulthood and lack the capacity to generate a GnRH/LH surge (75, 187). Likewise, neonatally castrated males show a feminized pattern of Kiss1 expression in the AVPV and estradiol administered during the neonatal critical period defeminizes Kiss1 expression, suggesting that in the normal male, testosterone exerts its effects on Kiss1 expression through an estrogen-receptor-dependent pathway (206).

Kiss1 expression in the ARC of the adult rodent is not sexually differentiated and thus not apparently dependent upon the perinatal sex steroid milieu (75). However, this generalization does not apply to the prepubertal animal, where it does appear that *Kiss1* and *NKB* (expressed in Kiss1 neurons) in the ARC are sexually differentiated. Prepubertal (postnatal day 15) rodents show a reduced rise in *Kiss1/NKB* expression in the ARC after gonadectomy (compared with females), and this phenomenon is associated with a more restrained postcastration rise in LH in the male compared with the female (for preliminary data, see Ref. 188). This sexually differentiated response to castration in the prepubertal animal does not occur in the adult, wherein both sexes manifest a parallel rise in *Kiss1/NKB* expression and LH (188). Thus, sex differences in the tempo of sexual maturation (females being earlier than males) may reflect a differential sensitivity to the steroid milieu in the prepubertal animal. This remains to be tested.

VII. Action outside the Hypothalamic-Pituitary Axis

The current literature on the kisspeptin signaling system has been focused primarily on the roles of kisspeptin in reproduction and tumor metastasis. However, some recent studies indicate that kisspeptin may serve additional physiological functions in the nervous system and beyond. Kiss1 and Kiss1r expression is not restricted to the neuroendocrine axis but can be found in a variety of organs, with widespread and divergent implications.

A. Hippocampus and amygdala

In 1971, Velasco and Taleisnik (189) described a modulating influence of the amygdala and hippocampus on gonadotropin release. More recently, *in situ* hybridization has revealed expression of *Kiss1r* in these two brain regions (2, 3, 6), although a clear role of kisspeptin in the limbic system is unclear. It is interesting to note that the expression of *Kiss1* mRNA in the hippocampus is increased by about 50% 2 wk after gonadectomy in the male rat (190), suggesting a possible neuroendocrine function in this area. *Kiss1r* is highly expressed in the granule cells of the dentate gyrus (6), and kisspeptin treatment enhances excitability of those cells (191). Thus, kisspeptin may play a role in various neurological processes, including cognition, regulation of neurogenesis, or pathogenesis of epilepsy (192). *Kiss1* mRNA in the amygdala is also regulated by testosterone in the male mouse (193).

B. Adrenal

The high levels of kisspeptin during pregnancy may affect maternal and/or fetal adrenal production of aldosterone. During the third trimester of pregnancy, Kiss1r protein is highly expressed in the neocortex of fetal adrenals, at a level significantly higher than in adult adrenals (194). Notably, kisspeptin increases aldosterone production in fetal adrenal cells and H295R adrenal cells, increases angiotensin II-stimulated aldosterone production, and increases the ability of the H295R cells to metabolize exogenous pregnenolone to aldosterone (194). These observations provide insight into the possible regulation of adrenocortical steroidogenesis and function of the human fetal adrenal gland during the latter stages of pregnancy.

C. Pancreatic islets

Recent evidence points to a relationship between pancreatic endocrine cells and kisspeptin. Pancreatic β-cells sense and respond to both short-term fluctuations and long-term changes in energy balance, and the majority of islet endocrine cells express high levels of Kiss1 and Kiss1r, which both colocalize with insulin and glucagon (195). Expression of both kisspeptin peptide and receptor within an individual islet cell suggests a local autocrine or paracrine (*vs.* systemic) mode of action for pancreatic islet kisspeptin. A stimulatory effect of Kiss1 on glucose-induced insulin secretion in mouse and human islets (195, 196) implies a regulatory role of kisspeptin in normal regulation of islet function. However, the importance of this system has yet to be established because humans with mutated *KISS1R* and mice bearing deletions of the *Kiss1r* and *Kiss1* (knockout mice) have not been reported to exhibit any apparent metabolic abnormality.

D. Ovary/oviduct

Evidence suggests that kisspeptin plays a role at the level of the ovary. Castellano *et al.* (197) report expression of *Kiss1* and *Kiss1r* in the adult rat ovary throughout the estrous cycle. It appears that levels of *Kiss1r* mRNA in the ovary remain relatively constant throughout the estrous cycle; however, levels of *Kiss1* mRNA fluctuate dramatically, with a sharp increase on the afternoon of proestrus, directly preceding ovulation (197). Furthermore, immunohistochemical analysis demonstrates the presence of Kiss1-ir in the rat ovary, particularly in the theca layers of growing follicles, corpora lutea, and interstitial gland (197). Collectively, these observations suggest that locally produced ovarian kisspeptin directly influences folliculogenesis, ovulation, and perhaps luteal function in rats, which may also apply to other animals, including humans and marmosets, where kisspeptin has been identified in the ovary (198).

The postovulatory events in female reproduction may also depend on locally produced kisspeptin; Gaytán *et al.* (199) have proposed a role for oviductal kisspeptin in the prevention of ectopic (tubal) implantation. The authors describe a regional-specific pattern of expression that appears to be cycle-dependent, with maximum expression at the time of proestrus/estrus and lower levels at metestrus/diestrus (199). Following the role of kisspeptin in regulating uterine implantation (200), kisspeptin expression in the oviduct may play a physiological role in preventing ectopic implantation in the rat (and perhaps other species as well).

E. Vasculature

Mead *et al.* (201) have explored a possible role of kisspeptin in the cardiovascular system of humans. They report expression of *KISS1R* mRNA in the aorta, coronary artery, and umbilical vein, and subsequently immunocytochemically localized KISS1 and KISS1R to the atherosclerotic plaque of the coronary artery. Utilizing RIA along with immunocytochemistry, the authors identified a potential source of kisspeptin in vascular endothelial cells, which could enable paracrine regulation of vascular tone (201). Furthermore, Kisspeptin-10, -13, and -54 can act as potent vasoconstrictors in isolated human coronary artery and umbilical vein, producing a response as robust as the response to angiotensin-II in the coronary artery (201). Collectively, these findings allude to a novel function of kisspeptin and its receptor in mediating vasoconstriction, especially in blood vessels prone to atherosclerosis, which yields important implications in the pathophysiology of cardiovascular disease and is perhaps even related to preeclampsia in pregnancy.

VIII. Closing Remarks: Challenges, Open Questions, and Future Directions

Kisspeptin is a peptide with a diverse and multifunctional nature, involving varied whole body physiological systems and acting at all levels of the reproductive axis— brain, pituitary, gonad, and accessory organs. Kisspeptin exercises a crucial role in stimulating GnRH, relaying steroid hormone negative and positive feedback signals to GnRH neurons, serving as a gatekeeper to the onset of puberty, and relaying photoperiodic information. Other less well-defined actions of kisspeptin may include a role in the control of insulin and/or glucagon secretion, perhaps local control of ovulation, and blocking ectopic implantation, to name a few. The field of "reproductive kisspeptinology" has blossomed and matured in the past 6 yr; however, much is yet to be learned and important questions remain unanswered. For example, what specific neurotransmitters and signaling molecules control kisspeptin secretion? What is the role of kisspeptin's cotransmitters in the ARC (dynorphin, NKB, and others?) in the regulation of GnRH secretion? How do circadian signals from the SCN interact with the kisspeptin neurons in the AVPV? What activates kisspeptin neurons at puberty? What is the molecular basis for sexual differentiation of kisspeptin neurons? What molecular form(s) of the various kisspeptin fragments represent the endogenously active molecule? What is the physiological significance of kisspeptin signaling outside of the hypothalamus? What are the molecular mechanisms by which estradiol inhibits the expression of Kiss1 in the ARC but induces its expression in the AVPV? How do progesterone and the PR influence Kiss1 gene expression? What are the electrophysiological properties of Kiss1 neurons in the AVPV and ARC? What is the functional significance of enhanced Kiss1 production during pregnancy?

Several investigational tools may aid in answering these questions. For example, generation of a mouse expressing green fluorescent protein under the *Kiss1* promoter (*Kiss1*-GFP), a *Kiss1*-cre mouse, a floxed *Kiss1* mouse, or a *Kiss1* ribo-tagged mouse could prove invaluable to answer these questions. Another frontier in kisspeptin biology is the development of novel ligands (antagonists and agonists) to the kisspeptin receptor. Such analogs could prove useful in the treatment of people with hypogonadotropic hypogonadism and other reproductive disorders (*e.g.*, precocious puberty, endometriosis, metastatic prostate cancer, and ovulation induction) and could even provide a novel strategy for hormonal birth control (for both males and females). Beyond this, kisspeptin has been implicated for a role in a variety of other physiological control systems (*e.g.*, metabolism, vascular biology, pregnancy, cancer)—thus, improved understanding of kisspeptin and its receptor may benefit scientific research across a wide swath of the physiological community, not just reproductive endocrinology. We eagerly await the next chapters in the kisspeptin story.

"*It doesn't matter how beautiful your theory is, it doesn't matter how smart you are. If it doesn't agree with experiment, it's wrong.*"

–Richard Feynman, physicist, Nobel laureate (1918–1988)

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