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Does the KNDy model for control of GnRH pulses apply to monkeys and humans?

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Abstract

There is now considerable evidence supporting the role of a subpopulation of neurons in the arcuate nucleus of the hypothalamus that co-express kisspeptin, neurokinin B, and dynorphin (abbreviated as KNDy neurons) as the long sought-after GnRH pulse generator. The "KNDy hypothesis" of pulse generation has largely been based on findings in rodents and ruminants, and there is considerably less information about the anatomical and functional organization of the KNDy subpopulation in the primate hypothalamus. In this review, we focus on the applicability of this hypothesis, and the roles of kisspeptin, neurokinin B and dynorphin in reproduction, to humans and non-human primates, reviewing available data and pointing out important gaps in our current knowledge. With recent application of drugs that target KNDy peptides and their receptors to therapeutic treatments for reproductive disorders, it is imperative we fully understand the primate KNDy network and its role in the control of GnRH secretion, as well as species differences in this system that may exist between humans, non-human primates and other mammals.

Keywords

kisspeptin; neurokinin B; dynorphin; gonadotropins; estradiol

Introduction

Over the last decade considerable evidence was developed based on work in rodents and ruminants supporting the hypothesis that a group of arcuate (ARC) neurons that contain kisspeptin, neurokinin B (NKB), and dynorphin (known as KNDy neurons) play a key role in synchronizing GnRH neural activity during episodic GnRH secretion (1,2). The original form of this hypothesis proposed that during generation of GnRH pulses, kisspeptin was the output signal from KNDy neurons to GnRH cells, with NKB and dynorphin acting as start and stop signals within the KNDy neural circuitry (3–5). Because estradiol (E_2) and

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testosterone inhibited kisspeptin expression in the ARC (6,7) it was also proposed that these neurons mediate the negative feedback actions of gonadal steroids on episodic GnRH/LH secretion (8). Subsequent tests of various aspects of this hypothesis, while generally supporting it, have also pointed to species differences among rodents and ruminants in the operation of this system (9) and led to some controversy as to their role in steroid negative feedback (10,11). In light of the recent reviews of this topic that emphasized work in rodents and ruminants (2,9), here we will focus on the evidence for, and against, the applicability of this model to humans and non-human primates. We will first briefly summarize the species differences among rodents and ruminants and then review the available data on the roles of kisspeptin, NKB, and dynorphin in controlling GnRH/LH pulses and mediating steroid negative feedback in monkeys and humans.

Comparison of data in rodents and ruminants

The strongest evidence that synchronous activity of KNDy neurons drives episodic GnRH/LH secretion comes from a recent optogenetic study in mice (12). Moreover, several lines of evidence from both rodents and ruminants support the following proposed roles for kisspeptin and NKB: 1) kisspeptin is the output signal from KNDy neurons that drives GnRH secretion during a pulse (13,14), 2) kisspeptin may also act on non-KNDy neurons in the ARC to stimulate GnRH/LH pulse frequency (15,16), and 3) NKB stimulates GnRH release primarily by increasing kisspeptin release from KNDy neurons (17).

On the other hand, there appear to be two major differences in the functioning of this system between rodents and ruminants. First, there is considerable redundancy among the three major tachykinins-receptor signaling complexes (NKB-NK3R, neurokinin A [NKA]-NK2R, and substance P [SP]-NK1R) in rodents so that NKA or SP can substitute for the loss of NKB to maintain LH pulses (18). Thus the combined action of antagonists to NK1R, NK2R, and NK3R are needed to inhibit LH pulses in ovariectomized (OVX) rats (19) and the stimulatory actions of NKB on the electrical activity of KNDy neurons in murine slice preparations (20). In contrast, selective NK3R antagonists readily inhibit LH pulses in OVX ewes (16,21) and selective agonists for NK1R and NK2R produce little, if any, stimulatory effect in sheep (22) and goats (23). Second, there appears to be a major difference in the role of dynorphin. The stimulatory actions of nor-binaltorphimine (nor-BNI), a selective antagonist to the dynorphin receptor (kappa opioid receptor, KOR) on pulse frequency in sheep (16) and goats (4), and the internalization of KOR in KNDy neurons during a pulse (24) provides strong evidence for the proposed inhibitory actions of this endogenous opioid peptide (EOP). In contrast, although there is evidence that nor-BNI can depolarize KNDy neurons after their optogenetic stimulation in vitro (25), this antagonist had no effect on either LH pulses in OVX rats (26,27) or the basal electrical activity of KNDy neurons in murine slices (20,28).

Initial studies demonstrating the inhibitory effects of testosterone and E_2 on kisspeptin expression in the ARC of rodents led to the hypothesis that these actions could account for the negative feedback actions of gonadal steroids (8), but subsequent studies questioned this conclusion. Specifically in male mice, castration decreased the acute in vitro electrical activity of KNDy neurons (28,29). However, more long-term electrical recordings in vitro

(30) and chronic monitoring of calcium transients in vivo (31) clearly support a negative feedback action of the testes on KNDy neural activity. Moreover, changes in NKB and dynorphin might contribute to these actions. Castration increased NKB expression in KNDy neurons (28,32) and gonadal steroids suppressed the in vitro response of these neurons to an NK3R agonist (33). These steroids also enhanced the inhibitory actions of a KOR agonist (33), but since castration decreases dynorphin expression in rodents (28), the role of this peptide in males remains unclear. There is similar controversy about the role of KNDy neurons in mediating E_2 negative feedback because knockout of ERα in kisspeptin neurons (KERKO mice) did not affect the ability of E_2 to inhibit LH secretion, but did block the inhibitory actions of this steroid on kisspeptin expression (10). On the other hand, the report (11) of fewer LH pulses on the day of estrus in normal mice than in KERKO mice (which are in constant estrus) suggested that ERα in KNDy neurons is necessary for estrogen negative feedback. However, this difference could reflect the absence of a progesterone peak on the day before sampling in KERKO mice (that do not have an LH surge) since progesterone secretion on proestrus in rodents is responsible for the slow pulse frequency on estrus (34,35). Finally, there is some pharmacological evidence supporting a role for dynorphin in estrogen negative feedback in rats (27,36), but as noted above this is inconsistent with the inhibitory effects of steroids on dynorphin expression in rodents (3).

The data on steroid negative feedback in ruminants is less extensive, but also more consistent. There is little information in males, but kisspeptin and NKB expression in the ARC do increase in castrated sheep (37). Data that a KISS1R agonist increased LH secretion in the presence, but not the absence, of testosterone in castrated goats is consistent with maximal endogenous kisspeptin signaling following castration (38), but no other pharmacological tests are available in male ruminants. In contrast, there have been a number of studies in ewes (described in several recent reviews), which are largely consistent with the hypothesis that E_2 inhibits GnRH/LH pulse amplitude by suppressing kisspeptin release from KNDy neurons, while progesterone inhibits GnRH/LH pulse frequency by increasing dynorphin release from the same cells (9,39,40).

Evidence for the proposed role of kisspeptin in control of GnRH/LH pulses in humans and other primates

Roles for kisspeptin and KISS1R in the regulation of the reproductive axis were first established by observations made in humans, where loss-of-function mutations in KISS1R were shown to result in normosmic congenital hypogonadotropic hypogonadism. These seminal studies revealed that inactivating mutations in the KISS1R gene resulted in partial or complete failure of puberty and infertility (41,42). Infusion of kisspeptin into human male and female subjects was shown shortly thereafter to significantly increase plasma LH, FSH, and gonadal steroids (43,44). That the KISS1R and its cognate ligand, kisspeptin, are obligatory elements of neuroendocrine circuits controlling GnRH release, puberty, and adult fertility was confirmed in a variety of mouse models, including studies of reproductive deficits in *Kiss1R*$^{-/-}$ and Kiss$^{-/-}$ mice (41,45).

Reduced basal serum LH levels and altered pulsatile LH secretory profiles have been consistent findings in individuals with loss-of-function KISS1R mutations, with loss of signaling rigorously documented in cell expression systems. Surprisingly, LH pulsatility appears to persist in these individuals at a relatively normal frequency, albeit greatly reduced pulse amplitude (41,46). By contrast, an inactivating mutation in *KISS1* was described in a large consanguineous family that was found to result in failure of puberty associated with a complete absence of detectable LH in serial blood samples (47). It is difficult to conclude from the latter study that the absence of LH pulses reflects an inoperative GnRH pulse generator, since residual pulsatility may occur at levels below the detectable limits of the assay. Nevertheless, the observations of severely reduced LH pulse amplitude and mean LH in the absence of either kisspeptin or its cognate receptor are consistent with the idea that kisspeptin signals through the KISS1R to mediate most of the synchronized pulse initiation signal to GnRH neurons. It is possible that low-amplitude pulses that persist in the absence of kisspeptin or KISS1R signaling reflect the capability of GnRH neurons to maintain an intrinsic pulsatile secretory output, as suggested in early experiments using cultured immortalized GnRH neurons (48). The ability of continuous kisspeptin infusions to restore pulsatile LH secretion in patients with NKB signaling deficiencies (49) may be explained by the emergence of an intrinsic GnRH neuronal pulsatility in the absence of endogenous kisspeptin-KISS1R signaling, and the amplification of that pulsatile output by kisspeptin. Alternatively, the KNDy cell pulse generator model derived from mouse studies may require modification as it pertains to humans, where a GnRH neuron-autonomous pulse generating mechanism may play a more predominant role.

It also remains unknown whether low-amplitude LH pulses that are observed in some of the foregoing affected patients reflect engagement of compensatory kisspeptin- or KISS1R-independent mechanisms during development. Residual GnRH secretory activity has been documented in *Kiss1R*$^{-/-}$ and Kiss$^{-/-}$ mice (50), and indeed appears to underlie the maintenance of fertility in animals in which kisspeptin neurons have been ablated early in development, attesting to the remarkable plasticity of neural systems governing pulsatile GnRH release. The induction of kisspeptin cell-specific gene ablation in adult animals will hopefully be possible in the foreseeable future, thereby allowing analyses that circumvent the confounding effects of compensatory developmental mechanisms.

Compelling evidence has been derived from studies of non-human primates that kisspeptin-expressing cells of the ARC comprise an integral component of the GnRH pulse generator. Early studies by Knobil and colleagues (51) used multiunit recording methods to characterize the electrophysiological correlates of GnRH pulsatility in the mediobasal hypothalamus (MBH) of rhesus macaques. While it has yet to be determined that these activity patterns arise from intermittent, synchronized activation of kisspeptin neurons, recent studies using optical imaging methods in mice make this scenario extremely likely (12). Microdialysis experiments in female rhesus macaques have revealed that kisspeptin is released in a pulsatile manner in the ARC-median eminence, and that fully 75% of kisspeptin pulses are temporally associated with GnRH pulse (52). Pulsatile injections of kisspeptin-10 induce corresponding GnRH-dependent LH pulses in juvenile male monkeys, where spontaneous GnRH release is virtually absent (53). Structural evidence for kisspeptin subserving GnRH pulsatility in the monkey is derived from the observations that kisspeptin

and GnRH fibers are intimately associated in the median eminence (54). It is not yet known if GnRH neurons in the monkey extend “dendron”-like processes into the median eminence where they are synaptically innervated by arcuate kisspeptin neurons, as has been shown to be the case in the mouse (55).

Additional support for kisspeptin-mediated pulsatility is provided by studies of kisspeptin release throughout puberty in the rhesus macaque. Puberty is initiated in monkeys by an activation of pulsatile GnRH release consisting of increased pulse frequency, amplitude and mean release level (56). Microdialysis experiments have revealed that kisspeptin pulsatility is increased in a parallel manner, viz. increased pulse frequency, amplitude, and mean kisspeptin level, throughout puberty in female rhesus monkeys (57). These findings are consistent with the foregoing model for the GnRH pulse generator in which kisspeptin neurons – likely KNDy neurons – comprise a pulse generating network that delivers kisspeptin intermittently to synaptic contacts with GnRH nerve fibers in the median eminence. During the quiescent juvenile period, the activity of the kisspeptin pulse generating network is suppressed, as is the downstream GnRH releasing terminal network in the median eminence. Upon activation by unknown pubertal initiation signals, kisspeptin pulsatility is increased and at least in part leads to the activation of the GnRH pulse generator. Additional evidence for the contribution of NKB release in this process is discussed below. It has been proposed that KNDy neurons play an integral role in the stimulation of GnRH pulses, but they are also passive with respect to the timing of pubertal onset (58).

There is even less data on the role of kisspeptin from KNDy neurons in mediating steroid negative feedback in humans and monkeys, but most of the available information supports this hypothesis. Thus kisspeptin expression in the MBH increases in older men (59), post-menopausal women (60) and peri-menopausal rhesus monkeys (61) when gonadal steroid levels are low. More directly, OVX increased and E_2 inhibited *KISS1* mRNA expression in monkeys (61) and both and E_2 and progesterone treatments decreased the number of *KISS1*-positive cells in the ARC in these animals (62). Similarly, testosterone replacement at the time of castration suppressed *KISS1* mRNA expression in the ARC, but not the preoptic area, of male monkeys (63). It should be noted, however, that exogenous estrogen increases the response of young (64) and post-menopausal (65) women to kisspeptin infusions, although this may relate more to the positive, than negative, feedback actions of this steroid (64).

In summary, a wealth of observations in humans and non-human primates supports the basic contention that a kisspeptin neuronal network in the ARC functions as integral components of the GnRH pulse generator and likely plays a role in steroid negative feedback. It remains to be determined whether 1) KNDy neurons serve as the entirety of the GnRH pulse generating mechanism, or some portion thereof, 2) non-kisspeptin cells systems contribute to pulse generation, 3) KNDy neurons solely innervate GnRH fibers in the median eminence or act in part via interneurons, and 4) GnRH neurons are capable of intrinsic pulsatility that may be amplified and/or entrained by kisspeptin signals.

Evidence for the proposed role of NKB in control of GnRH/LH pulses in humans and other primates

Perhaps the strongest evidence that NKB plays a key role in control of GnRH/LH pulses in humans was the discovery that mutations in NKB-NK3R signaling caused infertility in humans due to inadequate GnRH secretion (66). This observation also served as an important catalyst for the development of the KNDy hypothesis for GnRH pulse generation (1) and a number of subsequent studies tested this proposed role for NKB in humans and non-human primates. In contrast, there is almost no information in these species on the possible role of NKB in steroid negative feedback. Consequently, this section will focus on three questions relevant to the proposed role of NKB in generation of GnRH pulses: 1) What is the degree of overlap of NKB and kisspeptin expression in neuronal cell bodies? 2) Is there redundancy in tachykinin signaling similar to that seen in rodents? and 3) Do the stimulatory actions of NKB occur via, or independent, of kisspeptin release?

Co-localization of kisspeptin and NKB.

Although there is indirect evidence using in situ hybridization (ISH) that mRNAs for NKB and kisspeptin are found in the same neurons in post-menopausal women (60,67), there are no dual ISH studies using primate tissue, so this discussion will be limited to reports using dual immunohistochemistry (IHC). One important caveat applicable to interpretation of any study of co-localization is that expression of kisspeptin and NKB varies with hormonal status, and under some circumstances there can be changes in expression of only one of these peptides (68). It is thus important to examine co-localization under a variety of endocrine milieus whenever possible.

Two reports have described co-localization of kisspeptin and NKB in the rhesus monkey, both in males. In castrated adults 40–60% of kisspeptin perikarya in the ARC also contained NKB, while no cell bodies containing NKB alone were found (69). A similar percentage of kisspeptin cells containing NKB were observed in castrated infant (59%) and juvenile (61%) monkeys, although serum LH concentrations and the number of kisspeptin-positive cells were much lower in the latter (70). Cells containing only NKB were “seldom observed”. Thus, it appears that in the ARC of male monkeys there are approximately equal numbers of kisspeptin-only and kisspeptin+NKB neurons, while few, if any, cells contain only NKB. Whether this is also true in females is an important unresolved question.

There is considerably more data on co-localization of kisspeptin and NKB in humans (59,71–76), and several of these reports examined both cell bodies and kisspeptin or NKB-containing close contacts onto GnRH neurons (59,72–74). The latter, however, are difficult to interpret because the source of the input is not known (e.g. they could be from kisspeptin-only or NKB-only neurons with cell bodies outside the ARC). Although there are both sex-dependent (72) and age-dependent (59) changes in the number of kisspeptin-ir and NKB-ir neurons in the ARC, there is much less variation in co-localization of NKB in kisspeptin neurons (74) with a range from 73% (young men) (59,73) to 78% (old men and post-menopausal women) (59,75). In contrast the percent of NKB neurons containing kisspeptin varies from 33–36% in young men (59,73) to 68% in old men (59) and 67–

84% in post-menopausal women (74,75). These data indicate that there are likely three populations of kisspeptin and NKB-containing neurons in the ARC of humans and that there is a selective increase in kisspeptin expression (and hence higher co-localization with NKB) in older individuals.

Possible redundancy in tachykinin signaling.

The strongest evidence for redundancy among the three major tachykinins comes from reports that some patients with mutations in *TAC3* or *TACR3* show spontaneous recovery of reproductive function, and in some cases fertility, after hormonal treatments to induce development of the reproductive tracks and secondary sexual characteristics (77–79). Similarly, when examined, clear instances of episodic LH secretion have been reported after hormonal treatments, although pulse frequency is slower than in normal individuals (49,77,79). However, non-episodic LH patterns after treatment have also been reported (77,80,81) and these have always been observed before treatments (77). Although they have not been directly compared, there is suggestive evidence that the deficits in LH secretion may be more severe with *TACR3*, than *TAC3,* mutations. For example in one of the first reports, the maximum LH concentrations in three patients with *TACR3* mutations ranged from 0.15 to 0.2 IU/L compared to 2.4 to 3.8 U/L in three with mutations of *TAC3* (80); this group subsequently reported more frequent LH pulses in two patients with *TAC3* mutations than in two with them in *TACR3* (49). On the other hand, several males with mutations in *TACR3* had clear evidence of spontaneous improvements in reproductive function, although only 3 of 13 were fertile (77). Interestingly, this same study found no evidence of spontaneous recovery in four females with *TACR3* probands, while 3 of 4 females with *TAC3* probands recovered normal function (77). This difference, if confirmed in future work, points to a redundancy in tachykinin ligands, rather than receptors, as a likely explanation for this spontaneous recovery. If this is the case, then SP is the most likely candidate because it can stimulate LH secretion in men (82), is co-localized with kisspeptin and NKB in the human ARC (83) and expression of the mRNA for SP increases in post-menopausal women (67).

While genetic studies argue for redundancy in tachykinin signaling in humans, pharmacological work argues against it. Thus, two different antagonists that are specific for NK3R inhibit tonic LH secretion in normal men and women (84–86) as well as post-menopausal women (87) and patients with polycystic ovarian syndrome (PCOS) (88). This contrast with the data in rats, in which a specific NK3R antagonist had no effect on LH pulses in OVX animals (19). Similarly, most data in non-human primates point to a lack of redundancy in tachykinin signaling. There are very few SP-containing neurons in the primate ARC, although the number did increase with castration, and exogenous SP failed to stimulate LH secretion in male monkeys (89). More importantly, a NK3R antagonist inhibited LH secretion in castrated monkeys and suppressed estradiol levels and delayed (or blocked) the LH surge during the menstrual cycle of cynomolgus monkeys (90).

In summary, strong genetic data point to some redundancy in tachykinin signaling in humans, with SP likely able to substitute for NKB in some of these patients. In contrast, pharmacological data in both humans and non-human primates indicates that little, if any,

redundancy exists. Based on analogous situations in mice, the simplest explanation for this apparent paradox is that in normal individuals NKB-NK3R signaling is critical for pulsatile LH secretion, but when this signaling is disrupted redundant systems come on-line during in utero or post-natal development.

Interactions of kisspeptin and NKB in stimulating GnRH secretion:

Not surprisingly, there is little direct information on the relationship between kisspeptin and NKB in humans. There is one report that co-infusion of NKB decreased the stimulatory effects of kisspeptin on mean LH concentrations in men by about 20%, but NKB had no significant effect on LH pulse patterns in that study (91). The ability of exogenous kisspeptin to stimulate LH secretion in patients with mutations in *TAC* or *TACR3* (49,79) supports the hypothesis that NKB action "is proximal to kisspeptin" (49) in stimulating GnRH secretion. However, as discussed above, the possibility of redundancy in tachykinin signaling in these patients argues for caution in interpreting these data.

There is more direct evidence that NKB acts via kisspeptin in rhesus monkeys. In an early test of this relationship, down regulation of KISS1R in castrated juvenile males blocked the ability of senktide, a NK3R agonist, to increase LH, but suppression of NK3R had no effect on the stimulatory actions of kisspeptin (92). Furthermore, senktide increased kisspeptin concentrations in microdialysates of the MBH of both male and female monkeys (93,94). More recent work using reverse microdialysis to deliver agonists and antagonists to the MBH of rhesus monkeys has suggested a more complex relationship between kisspeptin and NKB (93,94). Infusion of p234, a KISS1R antagonist, blocked the stimulatory actions of senktide, on GnRH release in prepubertal male and pubertal male and female (post menarche, but before first ovulation) monkeys. In contrast, in prepubertal female monkeys this antagonist delayed, but did not completely block, the actions of senktide so that GnRH release increased shortly after termination of treatment with both senktide and p234. While these results are largely consistent with the hypothesis that NKB acts via kisspeptin, the experiments with the NK3R antagonist, SB222200, were not. This antagonist blocked the stimulatory action of kisspeptin in prepubertal males and pubertal females, but not in pubertal males and prepubertal females. Thus kisspeptin may act via NKB at some stages of pubertal development, but not at others. These results led to the proposal that there are interactions between kisspeptin-only and NKB-only neurons in the MBH of monkeys, both of which project to GnRH cells, and that these change over development (93), but there is currently no evidence for neurons that contain only NKB in monkeys. An alternative explanation is that in prepubertal males and pubertal females kisspeptin or NKB input alone to GnRH neurons is insufficient to stimulate GnRH release, so that both are required to increase GnRH secretion. It should be noted, however, that it is unclear whether GnRH neurons in primates contain NK3R (or KISS1R). Finally, there are two reports in humans that an NK3R antagonist had no effect on the acute response to kisspeptin administration, one in men (85) and the other in women (64).

Key gaps in knowledge.

In conclusion, there are some important gaps in our knowledge on the roles of NKB and kisspeptin in humans and non-human primates. Specifically, three critical questions arise

from this review: 1) Are there NKB-only neurons in the ARC of female rhesus monkeys? 2) Do GnRH neurons contain NK3R in humans or non-human primates? 3) Do the differences in effects of NK3R antagonists on the stimulatory actions of kisspeptin between humans and monkeys reflect species differences or are they due to the lack of studies in adult monkeys to compare with humans?

Evidence for proposed role for dynorphin in GnRH pulse secretion in humans and other primates

Early evidence in humans linked endogenous opioid peptides (EOPs) to an inhibitory role in the control of GnRH/LH secretion (95,96). In mammals, the EOP system consists of three ligand families: the endorphins, the enkephalins and the dynorphins (97). There are three main classes of EOP receptors that mediate the function of EOPs in the brain: δ, μ and κ, all of which are Gi/Go protein-coupled seven-transmembrane receptors (97,98). The main EOPs that are involved in the hypothalamic control of LH secretion are dynorphin and β-endorphin. Compelling evidence on the role of KNDy neurons in regulation of pulsatile LH secretion in rodents (3) and sheep (16) led to an interest in the inhibitory role of one EOP, dynorphin, and its κ-opioid receptor (KOR) on termination of the activity of the KNDy network-induced GnRH/LH pulse. Another EOP, β-endorphin which mainly binds to δ and μ receptors, is also believed to inhibit pulsatile GnRH secretion from the hypothalamus. Therefore, in the current section, we will discuss EOPs as inhibitory modulators of GnRH secretion in humans and non-human primates through: 1) anatomical distribution of dynorphin in relation to GnRH regulation; 2) the role of EOPs in the regulation of pulsatile secretion of GnRH/LH; and 3) the role of EOPs in mediating steroid negative feedback.

Colocalization of kisspeptin and dynorphin.

Early anatomical evidence described the distribution of dynorphin-immunoreactive (dyn-ir) cells in the human ARC (99). The hypothesis that KNDy neurons are the GnRH pulse generator (69,100,101), has raised the interest in the role of dynorphin in the ARC in regulation of LH pulses in mammals, and most importantly, in humans. However, current evidence indicates that the degree of colocalization of dynorphin with kisspeptin and NKB in non-humans primates and humans is limited (60,102,103). It is likely that dynorphin is co-expressed with kisspeptin in the ARC of non-human primates, but with lower prevalence (103). Evidence has shown a high co-expression of dynorphin and kisspeptin of 92% in the murine ARC (3) and 94% in the ovine ARC (101). There is only one study of this co-localization in non-human primates and it found, using IHC, that only 7% of kisspeptin-ir cells and 54% of kisspeptin-ir fibers in the ARC of female rhesus monkeys also co-localized dynorphin (103). The former is relatively low compared with over 40% NKB co-expression in kisspeptin cells in the ARC of the male primate (69). When interpreting these data, it should be noted that the much higher co-localization in kisspeptin-ir fibers may provide a more reliable index of co-localization if kisspeptin is rapidly transported out of the soma. Whether the low degree of co-localization is due to technical limitations of IHC in primates remains to be addressed.

Evidence from human studies has at times appeared to be conflicting on the co-expression of dynorphin and kisspeptin depending on sex and age. Low prevalence in dynorphin-ir cell bodies colocalized with kisspeptin was found in the ARC of young men (73), and post-menopausal women (76). However, the latter may be due to the loss of steroid negative feedback in older women. In contrast, using ISH, dynorphin neurons were later reported to be present in pre- and post-menopausal women, with prodynorphin (the precursor for dynorphin) mRNA detected in the ARC, and these cells showing a hypertrophy post menopause similar to kisspeptin and NKB neurons in the ARC (102). Unlike the elevation of NKB and kisspeptin gene expression, dynorphin mRNA expression decreased in the human ARC after menopause (102). Consistent with this, lower dynorphin expression levels in ovariectomized (OVX) ewes (104) and monkeys (105) have been reported. Thus, the rare colocalization in older women may reflect the loss of the stimulatory effects of sex steroids in dynorphin expression. Taken together, it appears that in both humans and other primates dynorphin is expressed in the KNDy population, but at fairly low levels. Whether the reported rare colocalization of dynorphin and kisspeptin negates the model of KNDy neurons in regulation of GnRH in primates should be interpreted with caution, because reproductive neuropeptide regulation varies between sexes, at different ages and under different hormone milieus.

Interactions of kisspeptin and EOP in control of GnRH secretion.

The inhibitory role of EOPs on LH secretion was reported long before the role of KNDy neurons as a upstream regulator of GnRH was discovered (106). Yet, there is no direct evidence in humans and non-human primates on the role of specific EOPs, especially dynorphin, in this action.

The opioid receptor antagonist, naloxone, may provide some evidence of the involvement of this pathway. Compelling clinical evidence has shown the lack of effects of naloxone on LH secretion in post-menopausal women (107–110) with absent ovarian steroid feedback. Consistent with this, in oophorectomized women (111), a 4 hr naloxone infusion had no effect on the LH secretion. These data contrast with work in younger women and men. In young women, administration of naloxone in the late follicular phase and luteal phase, but not early follicular phase, increased pulsatile LH release and LH pulse frequency (106). Similarly, in normal men, administration of naloxone (112) or naltrexone (91) increased the average LH level. In the latter study, co-infusion of naltrexone did not increase the effects of kisspeptin on mean LH concentrations, so that there may be separate mechanisms regulating LH secretion by EOP and kisspeptin in men. It should be noted that the effects of naloxone in young women and men may be confounded by EOP mediation of steroid negative feedback, but the recent report that naloxone increased LH pulse frequency in women with hypogonadotropic hypogonadism (79) indicates that naloxone can stimulate LH in the absence of steroid negative feedback. In summary, there is no direct evidence in primates that an EOP terminates GnRH/LH pulses. However, this may reflect use of relatively non-specific EOP antagonists such as naloxone. Evidence in sheep that naloxone doesn’t affect LH pulses in OVX animals (113), but nor-BNI, a specific KOR antagonist (114), increases the frequency of pulses in OVX sheep (16) and of MUA in OVX goats (4) indicates that more work, using a specific KOR antagonist, is needed in primates.

Role of EOP in steroid negative feedback.

Numerous lines of evidence suggest that EOP are involved in the negative feedback effects of sex steroids in animals as well as humans. In normal cycling women, EOP are strongly involved in the regulation of the menstrual cycle and the reproductive axis via inhibitory effects on hypothalamic pulsatile GnRH secretion (115,116). Moreover, the proposal that dynorphin is the EOP that mediates the negative feedback effect of progesterone is supported by compelling data from ewes. This is based on the following observations: 1) almost all dynorphin cells in the ARC express progesterone receptors (117); 2) prodynorphin mRNA expression decreased after OVX (104); and 3) nor-BNI increased LH pulse frequency in luteal phase ewes (118). Thus, a key question is whether dynorphin, or another EOP, conveys steroid negative feedback in monkeys, and more importantly in humans, but evidence in these species is not as abundant as in the ewe.

As mentioned above, dynorphin is co-expressed with kisspeptin in the ARC of primates, even though at low abundance (76,103). The most important early evidence implicating dynorphin in steroid negative feedback in humans is the ISH study showing dynorphin expressing cells have a hypertrophy (102) similar to that of kisspeptin and NKB cells in the human ARC after menopause (60). Moreover, in post-menopausal women, the number of neurons expressing prodynorphin mRNA in the ARC is reduced by 73% compared to pre-menopausal women (102); this contrasts with the elevated kisspeptin (60) and NKB mRNA (67) expression in these women. This reduction of inhibitory dynorphin mRNA in the absence of steroid negative feedback in post-menopausal women, pointed to a link between dynorphin and steroid negative feedback effects.

Studies using naloxone have also provided functional support for this proposal. In the early follicular phase of the normal menstrual cycle, when sex steroid levels are low, administration of naloxone did not alter pulsatile LH secretion (96,106). Consistent with this are the reports of lack of effects of naloxone treatments in post-menopausal women (110) and oophorectomized young women (111). In contrast, in women with a high progesterone milieu, whether under exogenous progesterone treatment (111) or endogenous progesterone secretion during the luteal phase (119), infusion of naloxone increased LH levels. Similar stimulatory effects of naloxone have been observed during the luteal phase of the monkey menstrual cycle (120–122) and in OVX animals with progesterone supplementation, but not OVX animals (113). These data strongly support the proposal that an inhibitory EOP mediates the negative feedback effects of progesterone. However, we should note that naloxone is a classic opioid receptor antagonist, which can bind to all three opioid receptors (123) so these data do not point to a specific EOP.

As noted above, changes in prodynorphin after menopause point to dynorphin, but that is also true for β-endorphin. Thus, based on ISH data, pro-opiomelanocortin (the precursor for β-endorphin) mRNA decreases in the human ARC after menopause (124). Moreover, progesterone administration increases β-endorphin in the hypophyseal portal blood of monkeys (125). In contrast, infusion of β-endorphin had no effect on LH secretion in post-menopausal women (107). This might be explained by data from female rats that steroids strongly upregulate the expression of the μ-opioid receptor (126), but this may not be the case in humans because no difference was found in μ-opioid binding potentials

using positron emission tomography during the menstrual cycle in women (127). In ewes, MBH microimplants of nor-BNI, but not aδ or μ-opioid receptor antagonist, increased LH pulse frequency in the luteal phase (118). Moreover, several lines of evidence in sheep (118) and rats (128) using nor-BNI, have indicated that dynorphin and its receptor KOR mediate this feedback effect but there is only modest support for this in primates. Taken together, although it appears that an EOP, possibly dynorphin, plays an important role in the progesterone negative feedback in primates, more evidence using specific EOP receptor antagonists may help answer the question.

There is also some limited data that EOPs may mediate the negative feedback actions of other steroids in humans. The possibility that an EOP is involved in estradiol inhibitory effects on LH secretion has been proposed based on the early findings that naloxone had no effect on LH secretion in post-menopausal women, but increased LH secretion when these women received estrogen treatment (109,111). Moreover, in normal cycling women, naloxone increased LH secretion in the late follicular phase under a high estrogen milieu, but not in the early follicular phase when estrogen levels are low (96,106). Similarly, naltrexone was able to overcome the inhibitory effects of estradiol treatment in normal men (112). As mentioned above, expression of precursor mRNAs for both dynorphin and β-endorphin decrease after menopause in women, which could reflect the low estrogen levels in these women. It thus seems reasonable to propose that an EOP may be involved in the negative feedback effects of estrogen in humans. There is evidence that dynorphin plays a role in estrogen negative feedback in rats (27), but EOPs do not seem to be involved in monkeys (120,122) or ewes (129). However, the role of dynorphin in this effect in women is unclear because of lack of evidence using specific EOP receptor antagonists. There is even less data on the possible role of EOP in androgen negative feedback in primates. As noted earlier, the stimulatory effects of naloxone (112) and naltrexone (91) in normal men support this role. In the only report directly testing this hypothesis in men, administration of dihydrotestosterone strongly inhibited LH secretion, and infusion of naloxone abolished the inhibitory effects of this androgen on LH levels (112). Therefore, the limited data available suggest that an EOP might at least be partly involved in the androgen negative feedback in men.

In summary, it is generally accepted that EOP have inhibitory effects on pulsatile GnRH/LH secretion in humans and non-human primates and, in particular, mediate progesterone negative feedback. Although some evidence suggests that dynorphin may possibly be the EOP mediating progesterone negative feedback, it is too early for a clear conclusion in primates before more work on the anatomical and physiological functions of dynorphin and KOR is done.

Key gaps in knowledge.

We know that a variety of endocrinological clinical conditions indicate that, in females, an EOP serves as a “brake” to GnRH/LH pulsatile secretion, the disturbance of which can result in diseases and infertility, such as PCOS. Moreover, it seems likely that dynorphin may be involved in the progesterone negative feedback in humans and other primates. However, there are several major gaps in our current knowledge on the role of EOP in

humans and non-human primates. These include: 1) Is KOR expressed in either KNDy and/or GnRH neurons? Preliminary evidence suggests that KOR is co-localized in NKB neurons of the female monkey ARC (Fig. 1), but this needs to be confirmed with co-localization of kisspeptin and dynorphin. 2) What is the extent of dynorphin colocalization with both kisspeptin and NKB in the monkey and human ARC? Is the reported low co-localization of dynorphin and kisspeptin due to technical reasons? 2) Does nor-BNI, or other specific KOR inhibitors, overcome the inhibitory effects of progesterone, or other gonadal steroids, in humans and non-human primates? 3) Does an EOP act through the KNDy network to produce the inhibitory effects on pulsatile LH secretion seen in humans and non-human primates?

Sex differences in KNDy neurons in humans and other primates

There is clear evidence for anatomical sexual dimorphism in the KNDy cell population in humans, with men having fewer kisspeptin and NKB-containing neurons than women (72). A similar sexual dimorphism in NKB, kisspeptin, and dynorphin expression is seen in KNDy neurons of sheep (130,131), but the situation in rodents is more complex. There is no sexual dimorphism in ARC kisspeptin expression in mice (132); in rats, IHC (133–136), but not ISH (137–140), studies found more kisspeptin neurons in the ARC, and NKB neurons in the caudal ARC (136), in females than in males. There is currently a lack of evidence as to whether the sex differences in KNDy peptides observed in humans are present in non-human primates, and this is a critical gap in the literature. It should be noted that sex differences in numbers of adult neurons could result from either a long-term change in gene/peptide expression and/or developmental effects on cell differentiation or survival. Hence, sexual dimorphism in humans could reflect differences in steroid negative feedback because the tissue was from older individuals so the women would have been post-menopausal and have increased expression of kisspeptin and NKB (60,67). The sexual dimorphism observed in KNDy neurons in sheep most likely reflects the organizational actions of androgens during pregnancy (130,131), and it remains to be seen whether prenatal steroids exert similar influences on the expression of KNDy peptides in the adult primate brain.

The functional significance of this sexual dimorphism is unclear, in part because most studies testing the KNDy hypothesis have been done in females (2), and there are few direct comparisons between male and female rats, although the LH response to senktide is lower in male than female rats (141). Interestingly, the limited available evidence for functional sex differences in this system comes largely from primates. For example, a NK3R antagonist (SB222200) blocked kisspeptin stimulation of GnRH release in female (93), but not male (94), post-pubertal rhesus monkeys. There are also sex differences in response to senktide and kisspeptin: the senktide-induced increase in kisspeptin and the kisspeptin-induced increase in GnRH are greater in female monkeys (93,94). In contrast, men are more responsive than women to kisspeptin-10 (44). One intriguing sexual dimorphism in humans is that kisspeptin-10 delays the next LH pulse in men (142), but not women (143), which raises the possibility of important sex differences in the effects of kisspeptin on the GnRH pulse generator. The mechanisms by which kisspeptin resets the pulse generator in the primate are unknown, although one possibility is that that this effect is mediated by

non-KNDy, *KISS1R*-expressing neurons of the ARC (144) which in the rat and sheep have been shown to stimulate GnRH/LH pulse frequency (15,16)

Conclusions

While evidence from anatomical, pharmacological, electrophysiological, and optogenetic studies in rodents and ruminants have strongly supported the "KNDy hypothesis" of pulse generation, important questions remain regarding the anatomical organization and physiological roles of this ARC subpopulation in the human and non-human primate brain. At a start, these include significant gaps in knowledge and potential differences between primates and other species in the co-localization/presence of the three KNDy peptides and receptors (Table 1). For example, the co-localization of NK3R and KOR within KNDy cells underlies one of the core elements of the KNDy hypothesis, namely the ability of NKB and dynorphin to act within a reciprocally interconnected network of KNDy cells as start and stop signals, respectively, for each GnRH pulse. NK3R and KOR colocalization has been reported in ARC kisspeptin cells in both mouse and sheep, albeit with different percentages (perhaps due to the use of IHC vs ISH, see NK3R in sheep, Table 1), but the co-expression of either receptor in monkey or human KNDy cells has yet to be examined. It would also be important to assess the co-expression of these two receptors in GnRH neurons. In addition, as mentioned above, differences in reported low co-localization of dynorphin and kisspeptin in the monkey ARC, and particularly in humans, may be due to technical reasons or attributable to sex, steroid or age differences; this needs to be rigorously re-examined with the same techniques and controls for those variables.

Beyond gaps to be filled with respect to the anatomical features of KNDy cells in primates, there are a number of questions regarding the physiological roles of KNDy peptides and receptors in the control of GnRH pulses. Some of these, such as whether kisspeptin drives, or is just permissive for, episodic GnRH release have yet to be completely addressed in any species. Others relate to possible species differences in the roles of these peptides. One of the latter is whether dynorphin (or another EOP) acts through the KNDy network to inhibit GnRH pulses in the human or non-human primate brain, as it has been shown to do in ruminants. Studies to explore whether nor-BNI, or other specific KOR inhibitors, overcome the inhibitory effects of progesterone or other gonadal steroids would be particularly worthwhile in testing the role of dynorphin as a stop signal for pulses in primates, regardless of whether that action is via KNDy or other cells. Finally, it is important to know whether the reported differences in effects of NK3R antagonists on the stimulatory actions of kisspeptin between humans and monkeys reflect species differences or age differences in the subjects.

Overall, there are a number of key questions regarding KNDy neurons in the primate hypothalamus that need answering in order to determine whether the KNDy model for control of GnRH pulses, as currently framed, can be applied to monkeys and humans. Given significant species differences between rodents and ruminants in the organization and function of this system reviewed above and elsewhere (9), it is entirely possible that the GnRH pulse generator in the primate is comprised of slightly different neurons and/or and neuropeptide/receptor components than other mammals. On the other hand, evidence

to date on the central roles of kisspeptin and NKB controlling GnRH secretion in primates suggests that the major elements may well be the same. With recent translation of findings stemming from the discovery of KNDy neurons to new therapeutic treatments for infertility (145), PCOS (88) and hot flushes (87,146), it is critical that we fully understand the KNDy network and its role in pulse generation in primates so that future clinical interventions are based on the most solid and rigorous scientific foundation possible.

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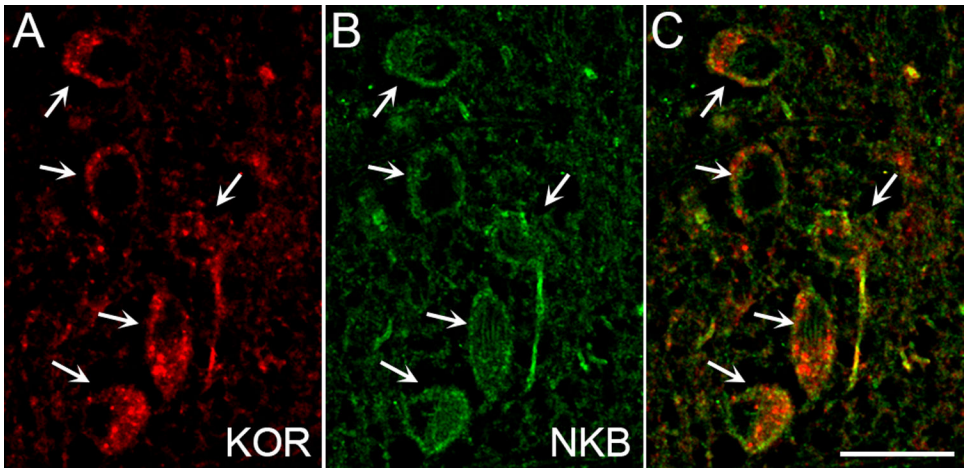

Figure 1.

Colocalization of KOR and NKB in the non-human primate ARC. Confocal image (1 μm optical section) of neurons immunolabelled for KOR (A, red), NKB (B, green), and the merged image (C) in an adult female *Rhesus macaque*. Arrows indicate dual-labeled cells.

Table 1.

KNDy peptide/receptor co-localization in rodents, sheep and primates

% ARC kisspeptin (KP) cells co-localizing:	Rodents	Sheep	Monkeys	Human
NKB	Mouse: 90%, OVX and OVX+E (3), 94%, castrated and castrated+T males (32); Rat: 97% (147)	80%, OVX+E, breeding season (80); 91%, OVX+E breeding season and anestrus (148)	40–60%, castrated adult males (45); 60%, infant and juvenile agonadal males (46)	71–78%, young and aged men and women (47), postmenopausal women (83)
Dynorphin	Mouse, 92%, OVX and OVX+E (3), 86%, castrated males (32)	94%, OVX+E, breeding season (80)	7% in KP cells but 54% in KP fibers, OVX adult female (82)	“Very rare”, young men (49), postmenopausal women (53)
NK3R	Mouse, 96%, OVX (3), 76%, castrated males (32)	47% (IHC), artificial follicular phase(149), 94% (ISH), luteal phase (He et al., SfN abstract, 2019)	N/A	N/A
KOR	Mouse: 20%, OVX and OVX+E females (3); 6%, castrated males (32)	98%, luteal phase, estrous cycle (150)	N/A	N/A