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Kisspeptins and the neuroendocrine control of reproduction

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1. ABSTRACT

Reproductive function, as essential for the survival of species, is under the control of a vast array of regulatory factors that ultimately modulate the release of GnRH. However, GnRH neurons lack the ability to directly sense most of these signals; hence, intermediate pathways are required. Kisspeptins have recently emerged as a pivotal piece in the reproductive brain, serving primarily as conduits for central and peripheral regulatory cues of GnRH release. Different populations of hypothalamic Kiss1 neurons have been described, which mediate either the positive or negative feedback of sex steroids in the sexually differentiated brain of rodents. Kisspeptins, however, are not the only recently-appointed contributors to this integrative process. Indeed, neurokinin B (NKB) and dynorphin have been described to co-localize within Kiss1 neurons at the arcuate nucleus in different species, and may contribute to the regulation of kisspeptin release. In this work, we provide a concise overview of the major reproductive headlines of kisspeptins, focusing on their role as mediators of sex steroid feedback and their interaction with key neurotransmitters, such as NKB and dynorphin.

2. INTRODUCTION

Reproduction is an energy-costly function, essential for the perpetuation of the species. As such, this is one of the most tightly regulated systems in the organism, whose development and function is under the control of a plethora of central and peripheral factors, many of which modulate the release of the hypothalamic decapeptide, *gonadotropin-releasing hormone* (GnRH); the ultimate driver of the activation of the gonadotropic axis (1). In recent years, our knowledge on reproductive endocrinology has experienced an unequivocal advance with the revolutionary discovery of the link between kisspeptins and reproductive function (2-4). The impact of this finding is only comparable to that of the discovery of GnRH itself. In fact, understanding the mechanisms mediating the fine regulation of GnRH release, as the masterpiece in the attainment and maintenance of the reproductive function (i.e. regulation of puberty onset and fertility in adulthood), has been a primary goal among reproductive biologists since GnRH was first described. Indeed, in spite of the leading role of GnRH in the control of the reproductive axis, the neurons producing this neuropeptide appear to

conspicuously lack the ability to directly sense the information conveyed by most of their major regulatory factors, thus suggesting the existence of intermediate afferent pathways. In this context, the emergence of kisspeptins in the reproductive arena in late 2003 has been fundamental for deepening our understanding on how these regulatory processes operate in order to enable proper sexual maturation and fertility in a wide range of mammalian and non-mammalian species.

Initially, kisspeptins were studied within the field of cancer biology as anti-metastatic molecules; its longer form received the name of *metastin* by virtue of its capacity to suppress tumor spread. These early publications identified metastin as a 54-amino acid (aa) product of the *Kiss1* gene (5), cleaved from a 145-aa precursor. These reports also identified other related fragments derived from the kisspeptin precursor, sharing the C-terminal RF-amide motif but with various aa lengths (14, 13 and 10-aa), therefore forming the family of kisspeptins (5, 6). Independently, in 1999, Lee *et al.* (7) described in the rat an orphan receptor, GPR54, a G-protein coupled receptor signaling mainly via phospholipase-C. This receptor was subsequently recognized as the putative kisspeptin receptor (8), and thus renamed as Kiss1r. This new terminology has been recently accepted within the scientific community (9), substituting the terms GPR54 and AXOR12/hOT7T175; the latter being initially used for the human orthologues (6).

3. KISSPEPTINS AND REPRODUCTION: *THE MISSING LINK*

In the last years, *kisspeptinology* has become an ebullient field in neuroendocrinology (10). An increasing number of publications from numerous laboratories worldwide have focused on deciphering the role of kisspeptins in the control of reproductive function. This was triggered by simultaneous reports from two independent groups showing that patients bearing loss-of-function mutations in the *KISS1R* gene displayed hypogonadotropic hypogonadism (HH) (2, 4). Importantly, this phenotype was replicated in genetically engineered *Kiss1* and *Kiss1r* knockout mice (*Kiss1*KO and *Kiss1r*KO, respectively (3, 4, 11, 12), evidencing a conserved role for this system across (mammalian) species. These initial findings strongly suggested already that the Kiss1/Kiss1r system is likely to operate as a nodal point controlling GnRH release, possibly acting as a transmitter of the actions of central and peripheral regulators on reproductive function.

Kisspeptins have been proven to potently stimulate luteinizing hormone (LH) and, to a lesser extent, follicle-stimulating hormone (FSH) release in a wide range of species, from mammals to fish, with documented stimulatory effects in some species, e.g. rodents, at the extremely low femtomolar range (13, 14). This action is thought to be mediated via activation of GnRH neurons. Indeed, many evidences support that kisspeptins act directly on GnRH neurons to stimulate GnRH release. First, GnRH neurons express *Kiss1r* as shown by *in situ* hybridization (15, 16) and lacZ reporter studies in a strain of *Kiss1r*KO mice (17). Second, GnRH antagonists are able to effectively block kisspeptin-induced gonadotropin release (13, 14). Third, kisspeptin increases the expression of *c-fos*, a marker of early cellular activation, in GnRH neurons (16). Forth, Kisspeptin induces very potent depolarization responses in GnRH neurons as measured by voltage recordings in mouse tissue (15), as well as the release of GnRH by hypothalamic preparations *ex vivo* (18) and to the portal blood system *in vivo* (19). Interestingly, the potent gonadotropin responses to kisspeptins are detectable both after central (intra-cerebroventricular) and peripheral (intraperitoneal and intravenous) administration (14, 20), suggesting that, at least partially, this action is conducted upon GnRH nerve terminals present in the median eminence outside of the blood brain barrier (BBB). Admittedly, however, the capacity of kisspeptins to cross the BBB is yet to be characterized and cannot be discarded either. Anyhow, the ability of kisspeptin to elicit the release of GnRH by mediobasal hypothalamic explants (devoid of GnRH cell bodies) *in vitro* (21) further supports a potential action of kisspeptins upon nerve terminals to stimulate GnRH secretion.

In addition, further evidence for the integrative role of Kiss1 neurons in the regulation of the gonadotropic axis comes from studies linking regulation of energy balance and reproduction. Thus, Kiss1 neurons express leptin receptor (Ob-R) (22). Leptin belongs to the pool of peripheral factors, originating from metabolic tissues (the adipose in the case of leptin), that transfer information about the magnitude of body energy stores to central neuronal networks governing different key functions, including reproduction (23). The levels of circulating leptin are proportional to the body fat mass; thus, situations of energy deficiency would fail to provide a proper leptin input to Kiss1 neurons, as reported in mice and rats lacking functional leptin signaling (*Ob-Ob* and *fa-fa*, respectively, (22, 24)). This leptin deficiency leads to a reproductive shut-down similar to the effect found during states of negative energy balance, i.e. fasting, undernutrition or diabetes (24-28). Noteworthy, the reproductive function in the latter paradigms in rodents can be rescued by means of exogenous administration of kisspeptin. This supports the notion that kisspeptin neurons act as a neuroendocrine funnel for the metabolic regulation of GnRH secretion.

4. NEUROANATOMICAL DISTRIBUTION OF KISS1 AND KISS1R

The distribution of *Kiss1* and *Kiss1r* mRNA and/or protein in the brain has been assessed in a wide range of species; however, due to space limitations and the scope of the review, in this work we will focus mainly on rodent data. In this context, several studies of *Kiss1* mRNA localization in the mouse have described two major areas of expression within the hypothalamus: the arcuate nucleus (Arc) and the anteroventral periventricular nucleus/periventricular nucleus (AVPV/ PeN) (13, 29). In addition, *Kiss1* mRNA was also found, to a lesser extent, in

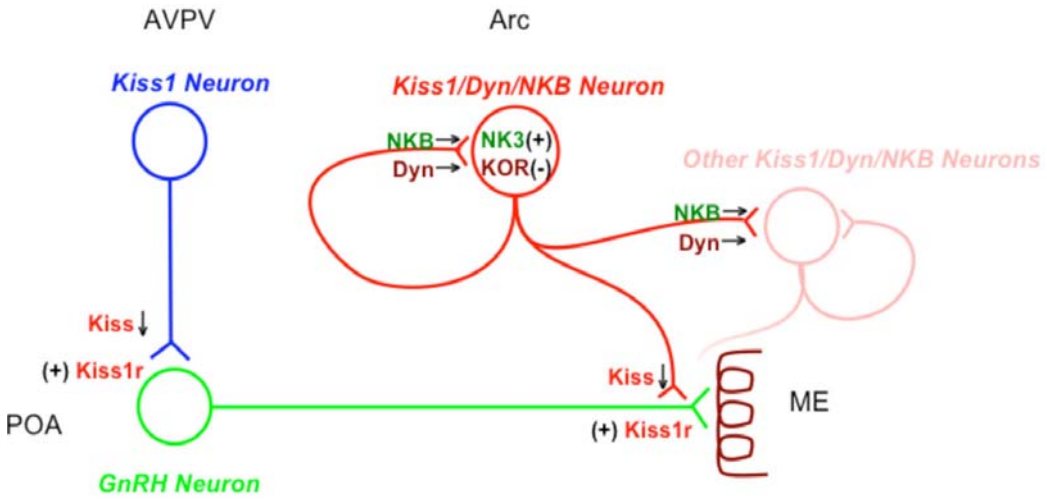

Figure 1. Schematic representation of interactions between the two major populations of Kiss1 neurons and GnRH neurons. Compelling evidence suggests that Kiss1 neurons at the AVPV mediate the positive feedback of sex steroids by acting upon GnRH neurons cell bodies. On the other hand, Kiss1 neurons in the Arc are considered the mediators of the negative feedback of sex steroids projecting to GnRH neurons axonal terminals. The population of neurons in the Arc has been suggested to play a key role in the generation of GnRH pulses. These Kiss1 neurons co-express *neurokinin B* (*NKB*), *dynorphin* (*dyn*) and their respective receptors (*neurokinin 3 receptor, NK3R*; and *kappa opioid receptor, KOR*). According to the model describe elsewhere (54), NKB would act autosynaptically on Kiss1 neurons to stimulate and synchronize the release of kisspeptin from the Arc. Then, dynorphin would be secreted leading to the inhibition of kisspeptin release, thus, shaping the kisspeptin -> GnRH pulses.

the preoptic nucleus, the amygdala and the bed nucleus of the stria terminalis (13).

Simultaneously, efforts have been made to describe the localization of kisspeptin at the protein level within the mouse and rat brain. In this context, the likely cross-reactivity of (some of) the initially available kisspeptin antibodies with other RF-amide peptides limited the validity of earlier studies. However, more recent assessments of kisspeptin immunoreactivity (Kiss1-ir) have offered a more detailed (and reliable) description of kisspeptin localization, although some degree of cross-reactivity with members of the RF-amide family cannot be totally excluded, even with the newer (and clearly improved) kisspeptin antisera. These analyses have documented an extensive level of overlapping between the mRNA and protein (29). Notably, these Kiss1-ir studies have remarkably contributed to the understanding of the physiology of Kiss1 neurons and their interaction with GnRH neurons. Indeed, studies in this front have evidenced that kisspeptin fibers contact GnRH cell bodies in the mouse (29), supporting a direct stimulation of GnRH release. In addition, studies in the monkey have demonstrated a predominant apposition of kisspeptin fibers coming from the medio-basal hypothalamus onto the GnRH terminals in the median eminence (ME) (30), which is currently considered as the principal region of interaction between kisspeptin –coming from the Arc- and GnRH neurons (Fig.1). This would indirectly point out that kisspeptin fibers from the AVPV preferentially interact with GnRH cell bodies at the level of the preoptic area; a contention that has been suggested on the basis of tracing studies (31) (Figure 1).

In addition, the distribution of kisspeptin fibers might indicate additional target areas within the hypothalamus, such as the retrochiasmatic area and the bed nucleus of the stria terminalis, as well as the supraoptic, paraventricular and arcuate nuclei (32), although such patterns of projections are yet to be fully characterized. However, kisspeptin projections within the Arc are likely to possess a significant importance, as there is a dense network of kisspeptin-ir fibers found to surround, and make contact, with Kiss1 cell bodies themselves (33), suggesting an autosynaptic feedback (*described below in more detail*).

Regarding the localization of Kiss1r, its characterization remains far more incomplete than that of its ligand, in part due to the absence of fully reliable antibodies anti-Kiss1r. Nevertheless, recent studies using a transgenic *Kiss1r* LacZ knock-in mouse model have revealed the presence of *Kiss1r* mRNA in the following brain areas: the dentate gyrus of the hippocampus, septum, rostral preoptic area (rPOA), anteroventral nucleus of the thalamus, posterior hypothalamus, periaqueductal grey, supramammillary and pontine nuclei, and dorsal cochlear nucleus (34). In addition, an increasing expression of this receptor in GnRH neurons of the rPOA has been reported along postnatal development (34).

5. ROLE OF KISSPEPTIN NEURONS AS MEDIATORS OF SEX STEROID FEEDBACK

The proper pattern of GnRH release is fundamental to attain reproductive capability. This release is subjected to a dual regulatory mechanism in most

species, depending on the sex and levels of sex steroids. On one hand, both sexes present a basal episodic secretion of GnRH that effectively stimulates gonadotropin release. In turn, this evokes the secretion of sex steroids from the gonads -estradiol (E_2) or testosterone (T)-, which feed back to the brain to down-regulate the release of GnRH. On the other hand, females also present positive feedback of sex steroids. Thus, the raising levels of E_2 that occurs at certain phases of the ovarian cycle stimulates GnRH neurons to acutely release a potent surge of GnRH which, once translated into an LH burst, the so-called *preovulatory surge* of GnRH/LH, triggers/facilitates ovulation. How GnRH neurons can discern between both antagonistic regulatory pathways elicited by the same signal (E_2) had remained largely as a mystery until the discovery of the Kiss1/Kiss1r system.

Compelling evidence suggests that Kiss1 neurons mediate both positive and negative feedback of sex steroids. Importantly, the vast majority of Kiss1 neurons (regardless their location in the brain) co-express estrogen receptor (ER)α (35). To note, GnRH neurons do not present ERα (36-38) (and only a subset do have ERβ (39), of as yet unknown physiological roles) evidencing the need for these neurons to rely on upstream modulators, e.g. Kiss1 neurons, to receive the message from sex steroids.

Notably, this dual role of Kiss1 neurons (mediating positive and negative feedback) depends on their neuro-anatomical distribution, which evidences the heterogeneity within these neurons in their ability to transmit sex steroid information to the GnRH neurons. Thus, substantial data from rodent (mouse, rat) studies point out that Kiss1 neurons in the AVPV/PeN of the female are mediators of the positive feedback of sex steroids to GnRH neurons, at least in these species (35, 40-42). Foremost, the expression of *Kiss1* in this area is up-regulated by E_2 in rodents, thereby driving a stimulatory input on GnRH release at the time of the LH surge. The importance of kisspeptin in this phenomenon is revealed by experiments of kisspeptin blockade with either kisspeptin antibody or a kisspeptin antagonist. Both experiments have shown a complete blockade of the preovulatory LH surge (40, 43, 44). Altogether, the above experimental evidence suggests a pivotal role of AVPV/PeN Kiss1 neurons in the generation of the surge-type of secretion of GnRH/ gonadotropins in the female. Strikingly, however, Dungan *et al.* have shown that E_2 is able to produce a modest LH surge in *Kiss1r*KO animals (45); a phenomenon that was rebutted shortly after by Clarkson *et al.* (46) using a different strain of *Kiss1r*KO mice. Whether this divergence is due to a difference in the LH-surge-generating protocol and/or the mouse strains remains to be solved. Furthermore, the characterization of the role of this eventual Kiss1-*independent* pathway in the generation of the GnRH/LH surge awaits specific investigation.

In contrast, Kiss1 neurons in the Arc of both sexes have been associated with the tonic basal drive of GnRH release. The expression of the *Kiss1* gene in these neurons is down-regulated by E_2, while it is up-regulated by gonadectomy (16, 35, 47). Animals lacking a functional Kiss1r show high levels of Kiss1 expression in the Arc (due to the absence of circulating sex steroids), but decreased levels of gonadotropins (hence, the reduced sex steroid secretion) (3). Consequently, we can infer that Kiss1 signaling emerging from the Arc accounts - at least partially- for the tonic control of pulsatile gonadotropin secretion and is a mediator for the negative regulation of gonadotropins by sex steroids.

In the above context, additional studies involving the generation of nucleus-specific knockouts of *Kiss1* expression are needed to parse out the genuine contribution of each kisspeptin neuronal population in the transmission of the sex steroid feedback. However, recent studies have offered already important hints to understand how different subsets of Kiss1 neurons can display such distinct responses to the same input, i.e. high levels of sex steroids. Thus, Gottsch and colleagues (48) have demonstrated that the mechanism of action of E_2 (via ERα) on *Kiss1* gene may differ depending on the anatomical location of the neuron. Therefore, Kiss1 neurons in the Arc respond to E_2 in an estrogen response element (ERE)-independent manner, the so called *non-classical* pathway, while Kiss1 neurons in the AVPV respond to E_2 through an ERE-dependent manner, the so called *classical* pathway.

In addition to the direct action of E_2 (or aromatized T) through ERα, Kiss1 neurons are likely to be regulated by additional steroid cues such as androgens and progesterone. Expression of androgen receptor (AR) has been documented in mouse Kiss1 neurons (35) and AR has been proven to exert an additional inhibitory signal upon these neurons in the Arc in the presence of T. As for the progesterone receptor (PR), its co-expression has been described in sheep and mouse AVPV/PeN (46, 49). The fact that PRKO mice are infertile (50) suggests an important role for PR signaling in the control of GnRH release, eventually, by acting on Kiss1 neurons. However, this hypothesis, as well as whether the Kiss1/PR co-expression is nucleus-specific in the mouse, needs to be experimentally evaluated.

6. INTERACTIONS OF KISS1 AND OTHER NEUROENDOCRINE SYSTEMS AT THE HYPOTHALAMUS: THE EMERGING ROLES OF THE NKB/NK3R SYSTEM

Kisspeptins may control GnRH release via both direct and indirect actions; the latter being suggested by the wide expression of Kiss1r in different hypothalamic areas and throughout the brain. Likewise, kisspeptins might also serve a role as mediators of the actions of a variety of neurotransmitters involved in the regulation of GnRH secretion. Indeed, in recent years, interactions between kisspeptins and other central regulators of GnRH neurons, such as GABA, glutamate, neuropeptide Y (NPY) and melanin-concentrating hormone (MCH) have begun to be documented, although the physiological relevance of such interplay is yet to be determined.

Also recently, it has become evident that Kiss1 neurons in rodents display heterogeneous features depending on their cerebral (hypothalamic) location. This

heterogeneity is also reflected by a distinctive pool of neurotransmitters being co-expressed in Kiss1 neurons in the AVPV, as compared to the ones co-expressed in the Arc. Thus, Kiss1 neurons in the AVPV of the mouse particularly co-express *tyrosine hydroxilase (TH)* mRNA (51), whose relevance is still unknown. Noteworthy, however, the AVPV population of Kiss1 neurons in the rat does not co-express TH (52), which indicates species differences in the physiology of this subset of Kiss1 neurons.

Likewise, Kiss1 neurons in the Arc are unique in that they co-express a specific, and to date larger, number of identified co-transmitters. In this sense, Goodman *et al.* (53) described the co-expression of *dynorphin A* (*dyn*) and *Neurokinin B* (*NKB*) in Kiss1 neurons of the ewe mediobasal hypothalamus using immunocitochemistry (ICC). Importantly, this phenomenon is conserved in different species. Thus, vast co-localization of the messengers of these three neuropeptides has been recently demonstrated in the Arc of the mouse by *in situ* hybridization (ISH) (54). This co-expression has also been shown in the goat (55) and there are evidences to believe that the NKB/dyn neurons described in rats (33) are in fact Kiss1 neurons. Of note, co-expression of NKB receptor (*neurokinin 3 receptor, NK3R*) and dyn receptor (*Kappa opioid receptor, KOR*) has been also documented in these Kiss1/NKB/Dyn neurons in the mouse (54). Furthermore, the expression of these genes at this hypothalamic nucleus is down-regulated in the presence of E_2, similar to *Kiss1*. Altogether, the above data disclose a greater degree of complexity of Arc Kiss1 neurons, suggesting the potential involvement of NKB and dyn in common regulatory pathways with kisspeptins.

Understanding how these neuropeptides act to modify kisspeptin release is currently a matter of intense research. Dyn is an endogenous opioid peptide produced in a wide range of brain areas with known inhibitory actions upon gonadotropin release in a number of species (56, 57). On the other hand, recent studies have proven a crucial role of the NKB/NK3R system in the control of reproductive function. Humans bearing loss-of-function mutations in either *TAC3* gene (encoding NKB in humans) or *TAC3R* (encoding NK3R), suffer hypogonadotropic hypogonadism and infertility (58). As potential call of caution, the as yet limited evidence gathered from the existing NK3R knock out models show these animals are fertile (59). Nevertheless, a detailed study of the different aspects of reproductive function of these animals, from puberty onset to senescence, is yet to be performed. In any event, recent experiments in rats have attested a stimulatory action of the NKB agonist, senktide, on LH release under physiological levels of sex steroids. This action is conducted, at least in part, through the stimulation of Kiss1 cells in the Arc, as shown by a significant increase in *c-fos* expression after senktide treatment (Navarro *et al. paper in preparation*).

While the field is rapidly moving, the existing data suggest that dyn, NKB and kisspeptin, acting as accomplices in the same neurons in the Arc, are involved in two crucial events for the reproductive viability of the animal. First, as stated above, they coordinate the transmission of the negative feedback of sex steroids. Second, Kiss1/NKB/dyn neurons might be directly implicated in the generation of GnRH pulses, an essential phenomenon for the maintenance of the reproductive function. An appealing possibility, that needs to be fully validated, is that autosynaptic stimulatory inputs of NKB upon Kiss1 neurons in the Arc are essential to drive the release of kisspeptin. In addition, the presence of a dense complex of NKB fibers surrounding Kiss1 cells in the Arc would support a potential synchronizing role of NKB signaling in the coordinated control of GnRH release. In turn, dyn would be released by the same neurons, evoking a decrease in kisspeptin secretion and, therefore, shaping kisspeptin pulses (Fig.1). Recent studies in monkeys (60) have demonstrated that kisspeptin release is pulsatile, supporting this model. Moreover, using recordings of multiunit activity (MUA) volleys in the mediobasal hypothalamus of goats, Wakabayashi *et al.* (55) have elegantly documented that these volleys (likely derived from the activity of Kiss1 neurons) are stimulated by exogenous NKB (and the dyn antagonist, norBNI) and suppressed by dyn. Notably, these volleys were paralleled by LH secretory bursts, adding further support to the predicted model of Kiss1 neurons in the Arc as essential components of the GnRH pulse generator.

7. CONCLUSIONS AND FUTURE LINES

The intense research generated after the discovery of kisspeptins as putative regulators of reproductive function has contributed to open up a new era in neuroendocrinology. However, while the role of kisspeptins in the stimulation of the gonadotropic axis has been extensively studied, the mechanisms governing kisspeptin release are still uncertain. In this context, further progress in *kisspeptinology* awaits the development of new genetically engineered mouse models that will allow broadening the knowledge of Kiss1 neuron physiology. Thus, there is a need for models expressing markers of Kiss1 location in living tissue, e.g. Kiss1-EGFP-expressing neurons, in order to perform electrophysiology studies on them. These analyses will offer invaluable information about direct responses of Kiss1 neurons to other neurotransmitters and endocrine regulators. In addition, conditional *cre*-recombinase (*Kiss1*-cre) and floxed (*Kiss1*-loxP) animals will permit cell-specific manipulations of co-expressing genes within Kiss1 cells. Therefore, substantial technological/ methodological advancements are expected in this front that may help featuring the signals and mechanisms whereby kisspeptin release is governed.

In close connection, further efforts are foreseen in the characterization of the interactions of kisspeptins with other putative regulators of GnRH release. Without doubt, recent identification of the co-expression of Kiss1, NKB and dyn in discrete neuronal populations of the Arc in a diversity of species has drawn quite some attention, and it is anticipated that considerable attention will be devoted to elucidate the precise mechanism whereby (and the individual roles of) NKB, dyn and kisspeptin exert the precise control of GnRH secretion. Again, some of the

methodological advancements listed above will contribute, together with expression analyses and pharmacological tests, to unveil the physiological roles of these Kiss1/NKB/Dyn neurons in the central control of reproduction.

In sum, we have provided herein a concise view of the state-of-the-art in some areas of kisspeptin physiology. Special emphasis has been made in summarizing our current knowledge on how Kiss1 neurons participate in the control of gonadotropin secretion by mediating negative and positive feedback effects of sex steroids. In this context, the emerging roles of NKB and dyn as accomplices of kisspeptins in the fine tuning of GnRH release have been highlighted and discussed. While the relevance of NKB and dyn in the control of different aspects of reproductive function remains to be fully elucidated, it is reasonable to predict that further efforts will be made in the coming years to elucidate their involvement in pivotal events such as sexual differentiation of the brain, the onset of puberty and its gating by energy status; phenomena on which kisspeptins have been suggested to play very prominent roles. Overall, it is expected that better characterization of the functions and modes of action of kisspeptins, and their co-regulators, will provide the scientific ground for the rational development of new contraceptive methods and novel strategies for the treatment of infertility, endocrine-dependent tumors and/or disorders of maturation of the reproductive axis.

8. ACKNOWLEDGMENTS

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