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Review

The GnRH Pulse Generator

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Abstract: The pulsatile secretion of hormones is an efficient way of coding a large variety of chemical messages. The GnRH pulse pattern determines which gonadotropin is released when and at what concentration, prescribing a detailed set of instructions to the gonads that produce changes in the steroid hormone milieu. Although GnRH neurons possess some inherent rhythmicity, they are diffusely situated within the hypothalamus and in isolation are only capable of generating physiologically irrelevant messages, hence a synchronization module exists upstream. The identity of the neural unit comprising the GnRH pulse generator is now generally thought to include KNDy neurons in the arcuate nucleus. These neurons coexpress the neuropeptides kisspeptin, neurokinin B and dynorphin A, as well as other transmitters, and are in intimate contact with the GnRH network. The GnRH pulse generator's function is the precise control of GnRH neuron excitability, coordinated activation, stimulation of neurosecretory events, modulation of gene transcription and the mediation of the negative feedback effect of gonadal steroids. Additionally, the GnRH pulse generator is an ideal venue for the integration of various sensory and homeostatic cues that regulate reproductive functions. In this chapter we provide a historical perspective of the elegant science that sparked interest in the central mechanisms underlying the functions of the reproductive system, explain how hypotheses surrounding GnRH pulse generation have evolved and describe the current state of knowledge within the dynamic field of GnRH pulse generator research.

Keywords: GnRH pulse generator; HPG axis; GnRH; LH; hypothalamus; kisspeptin; neurokinin B; dynorphin A; KNDy neurons; electrophysiology; neuroendocrinology; reproduction

1. Introduction

Reproduction is essential for the survival and evolution of all life forms on earth. Complex physiology underlies this process, particularly in higher organisms reliant on sexual reproduction. The ability of such organisms to effectively respond to physiological and environmental signals in order to tightly regulate the timing of reproductive events to ensure optimal reproductive outcome, and thus preserve the gene pool, has evolved over many millennia. Mammalian reproduction is under the stringent control of the hypothalamo-pituitary-gonadal (HPG) axis. Grossly, it resembles a macrofeedback loop that incorporates hypothalamic neural processes, endocrine cells of the anterior pituitary and the gonads, which are in communication through hormonal signals.

The major hypothalamic effector within the HPG axis is the neuropeptide gonadotropin-releasing hormone (GnRH), which is secreted from axon terminals in the median eminence (ME) by a population of neurons scattered throughout the preoptic area (POA) and hypothalamus. This secretion occurs in a pulsatile fashion [1], the dynamics of which vary according to reproductive status [2]. GnRH is released into the hypothalamic-hypophyseal portal circulation enabling the hormone to rapidly reach the anterior pituitary gland, where the activation of GnRH receptors on specialized gonadotrope cells leads to the secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), into the systemic circulation. Low-frequency GnRH pulses have a tendency to release FSH, whereas high-frequency GnRH pulses preferentially stimulate LH release [3]. The gonadotropins, in turn, regulate gonadal steroid synthesis and secretion.

In the female, the synthesis of estrogens, the most prominent and bioactive of which is 17β-estradiol (E_2), by ovarian granulosa and theca cells, is stimulated by the gonadotropins. Progesterone (P_4) synthesis by the corpora lutea, is likewise increased by elevated circulating levels of gonadotropins. The reproductive steroids act as regulatory signals influencing a diversity of processes throughout the body by altering the transcription of susceptible genes. The pulsatile neurosecretion of GnRH is itself highly sensitive to the effects of gonadal steroid signaling. Low levels of E_2 inhibit GnRH pulse amplitude, while P_4 restricts GnRH pulse frequency between ovulations [4]. High circulating concentrations of E_2, generated by the pre-ovulatory follicles, are stimulatory to the activity of the GnRH neural network [5]. The resulting marked increase in GnRH secretion [6,7] elicits an LH surge which triggers ovulation.

In the male, LH and FSH control the release of testosterone from Leydig cells of the testes, some of which is reduced to dihydrotestosterone or aromatized into E_2. The collective functions of gonadal steroids in adult males include restraint of GnRH secretion, maintenance of the male reproductive tract and spermatogenesis, thermoregulation and modulation of behaviour. Our

expertise is primarily in the female reproductive system, hence in this chapter we shall focus essentially on the research addressing female reproduction, while acknowledging critical sex differences.

2. The GnRH Pulse Generator Hypothesis

The development of a sensitive radioimmunoassay for the measurement of circulating LH levels [8] allowed the study of the pulsatile pattern of gonadotropin secretion [1]. LH is released from the anterior pituitary in regular bursts, during which plasma levels are rapidly elevated and then gradually decline with a half-life of 10–15 min, giving rise to a characteristic pulse-like profile. The unrestrained frequency of LH pulses differs between species: e.g. in the ovariectomized (OVX) monkey they occur approximately hourly [1] but are considerably more frequent (every 20 min) in OVX rats [9]. The subsequent discovery of GnRH as the putative gonadotropin-releasing factor [10,11] sparked research into its effects on the pituitary. It was soon shown that application of GnRH in a pulsatile, but not continuous, fashion sustains episodic secretion of LH [12–14]; in fact continuous GnRH stimulation abolishes secretion of both LH and FSH [13], an action that has been used extensively in clinical settings. Shortly after a radioimmunoassay for GnRH was introduced [15], GnRH pulses monitored in pituitary portal blood were demonstrated to occur at the same frequency as pulses of LH in the systemic circulation in the monkey [16] and the rat [17], and simultaneous episodes of GnRH and LH secretion were observed in the ewe [18]. Absolute correspondence between GnRH and LH pulses in the cerebrospinal fluid and systemic circulation, respectively, was then confirmed in the conscious OVX monkey [19].

In spite of the neural circuitry responsible for the rhythmic discharge of GnRH remaining unknown, the term ‘GnRH pulse generator’ has been used to describe this central neuroendocrine oscillator since the 1980s. To locate the GnRH pulse generator, various regions of the hypothalamus have been deafferentated or lesioned in a range of animal models. Deafferentation of the mediobasal hypothalamus arrested the estrous cycle in ovary-intact rats [20], but did not prevent the pulsatile secretion of LH in OVX rats [21] and monkeys [22]. In contrast, lesions of the hypothalamic arcuate nucleus (ARC) in the OVX monkey [23] and OVX rat [24] have been shown to cease pulsatile gonadotropin release. Moreover, synchronized bursts (or ‘volleys’) of multiunit electrical activity (MUA) recorded from the ARC were shown to coincide invariably with LH pulses in OVX monkeys [25], rats [26], ewes [27] and goats [28]. Taken together, these data implicate the ARC as the site of the GnRH pulse generator.

Since in primates the ARC contains a major population of GnRH neurons [29], it was postulated that GnRH neurons might possess inherent rhythmicity. More direct evidence for this proposal was obtained from studies of immortalized GnRH neurons [30–33], which release GnRH episodically under some circumstances. More recent *in vitro* studies revealed that cultured GnRH cells are capable of synchronized oscillation of intracellular calcium ion concentrations ($[Ca^{2+}]_i$) at a

frequency similar to that of pulsatile GnRH release [34,35]. Rhythmic calcium current synchrony was also observed between GnRH neurons and neuroglia isolated from the olfactory placode of the mouse [36] or monkey [37,38], which might explain how GnRH neurons are able to synchronize their activity despite their diffuse distribution in the mammalian brain [39,40]. Recently, there has been some interesting work on the control of GnRH secretion from human GnRH-secreting neuroblasts [41–43] and primary GnRH neurons [44], but these studies have not examined the mechanisms underlying episodic GnRH secretion. However, the rodent ARC is devoid of GnRH neurons [40], yet MUA volleys coincident with LH pulses are consistently recorded from this nucleus [26,45–49]. Furthermore, pulsatile LH secretion persists in OVX rats in which the mediobasal hypothalamus (MBH) that does not contain GnRH neurons was surgically separated from the rest of the brain [21, 50], and, although cultured mouse GnRH neurons showed spontaneous $[Ca^{2+}]_i$ oscillations, their frequency was inconsistent with that of GnRH pulses in the mouse [51].

The evidence for inherent rhythmicity of GnRH neurons does not necessarily conflict with the notion of an external GnRH pulse generator. Regardless of apparent differences in GnRH neuroanatomy between rodents and primates, all reproductively advanced species require a neural interface between the many intrinsic and extrinsic factors that regulate reproductive function. The ARC contains a wealth of highly interconnected neuroendocrine and centrally-projecting neuronal groups involved in homeostatic regulation, and is exquisitely permeable to signals arriving via the third cerebral ventricle [52]. It is thus not surprising that the current model of GnRH pulse generation is focused on neurons within this nucleus. To this end it seems reasonable to propose that although GnRH neurons are likely able to independently generate a synchronous rhythm, it is the GnRH pulse generator within the ARC that is responsible for fine-tuning the oscillatory activity of GnRH neurons in response to diverse regulatory signals.

3. Development of the KNDy Hypothesis for Pulse Generation

As discussed above, the evidence supportive of the notion that GnRH neurons are inherently capable of rhythmic synchrony and pulsatile peptide neurosecretion without requiring external inputs [31,34,37,38,53,54] led to the hypothesis that GnRH neurons are autonomous hypothalamic regulators of the HPG axis. Not surprisingly, therefore, cases of isolated normosmic hypogonadotropic hypogonadism, a disorder characterized by perturbed reproductive maturation and low gonadotropin levels, in patients with normal GnRH and GnRH receptor function were described as idiopathic. Hence, the seminal discovery of inactivating mutations in the gene *Kiss1r* encoding the putative receptor of the neuropeptide kisspeptin as a novel cause of hypogonadotropic hypogonadism in humans [55,56] attracted major interest in the field of reproductive endocrinology and led to numerous studies demonstrating that kisspeptin directly activates GnRH neurons in many species.

Kisspeptin, encoded by the *Kiss1* gene, was initially described as a suppressor of metastasis in malignant melanoma [57]. The previously orphan G protein-coupled receptor, GPR54, was

subsequently identified to preferentially bind kisspeptin [58–60] and is now more commonly referred to as Kiss1r. The expression profiles of *Kiss1* and *Kiss1r* mRNAs within the placenta, testis [59,60], as well as the pituitary, amygdaloid complex and the hypothalamus [61], are commensurate with kisspeptin's role as a regulator of reproductive function. Two main populations of kisspeptin neurons have been identified: one in the ARC and one situated more rostrally within the hypothalamus, but there are subtle species differences in the localization of the latter. Rostral hypothalamic kisspeptin neurons are concentrated in the anteroventral periventricular area (AVPV) and surrounding nuclei of the rostral periventricular region of the third cerebral ventricle (RP3V) in rodents [62], and scattered in the preoptic and periventricular areas in sheep [63] and humans [64], respectively [65] (Figure 1).

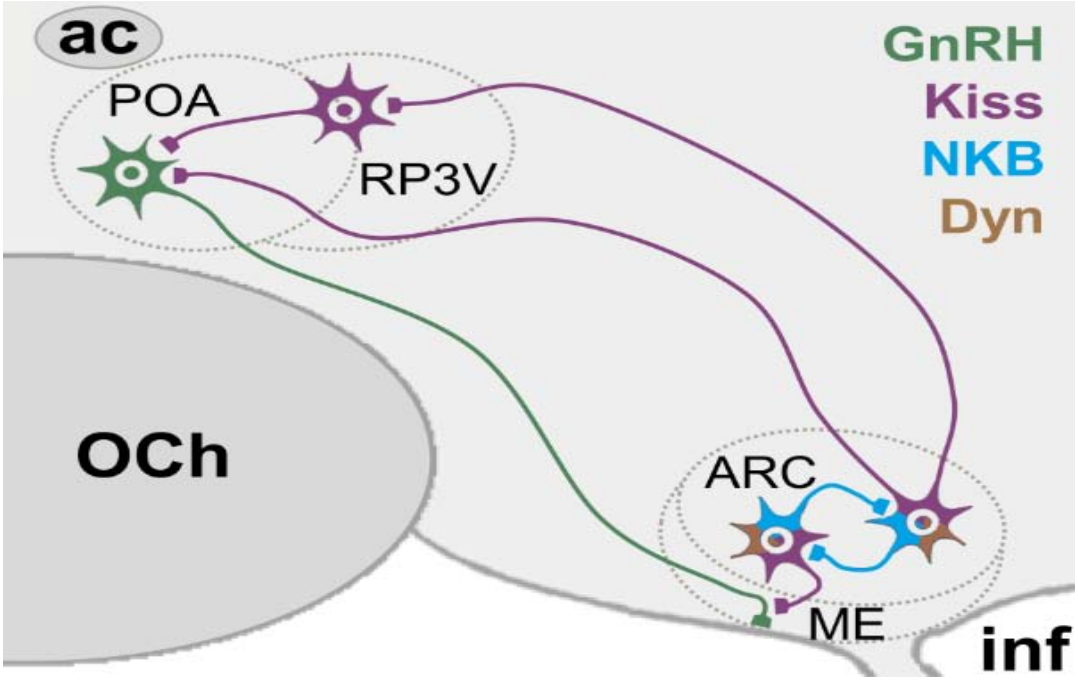

Figure 1. Schematic representation of connectivity between neurons implicated in the GnRH pulse generator. The arcuate nucleus (ARC) contains a population of neurons that coexpress the neuropeptides **K**isspeptin (Kiss; purple), **N**eurokinin B (NKB; blue) and **Dy**norphin A (Dyn; brown), and are therefore commonly known as KNDy neurons. A further population of kisspeptin neurons is found in the preoptic area (POA) in sheep and humans or rostral periventricular region of the third ventricle (RP3V) in mice and rats. Both populations of kisspeptin neurons innervate GnRH neurons that project from the POA to the median eminence (ME). In addition, KNDy neurons synapse with terminal processes of GnRH neurons in the ME. The integrated GnRH-KNDy system is hypothesized to resemble the neural substrate of the GnRH pulse generator, while the role of the rostral kisspeptin neurons is predominantly in the genesis of the preovulatory GnRH/LH surge. NB: The major population of hypothalamic GnRH neurons is found in the ARC in primates. Further, unlike

in other species, in which KNDy neurons project directly to GnRH perikarya, KNDy neurons are not known to project to GnRH cell bodies in the POA in rodents, and instead innervate RP3V kisspeptin cells that synapse with GnRH perikarya. For detailed discussion see Lehman *et al*., 2013. ac, anterior commissure; inf, infundibular recess; OCh, optic chiasm.

Besides **k**isspeptin, the neuropeptides **n**eurokinin B (NKB) and **dy**norphin A (Dyn) are coexpressed within the ARC population of sheep [66], but not the more rostral kisspeptin population; to easily distinguish this unique ARC population from other neurons containing these peptides, they are now widely referred to as KNDy neurons [67]. KNDy neurons have since been identified in several other species, but there are some differences in the extent of colocalization depending on species and endocrine condition [67]. Despite evidence that GnRH neurons can synchronize their activity and autonomously sustain pulsatile GnRH release, the fact that *Kiss1r* mutations impair reproductive function illustrates that GnRH neurons are not lone players. The early observation that ARC lesions abolish pulsatile secretion of LH [23] and that the same effect is achieved by intra-ARC infusion of a Kiss1r antagonist [68] suggest that KNDy neurons, which do not contain GnRH, constitute at least part of the neural substrate of the GnRH pulse generator. Other evidence supporting this hypothesis, which will be discussed in detail below, include: (1) NKB signaling, like kisspeptin signaling, is essential for fertility in humans; (2) KNDy neurons project to GnRH perikarya and terminals, (3) ARC MUA volleys can be recorded if electrodes are adjacent to KNDy neurons, but not in nearby tissue, and (4) KNDy neurons are extensively interconnected so that they may function as an integrated network (Figure 1). In the remainder of this chapter we will first consider the roles of KNDy peptides in the generation of GnRH pulses and the closely related negative feedback actions of ovarian steroids that modulate episodic GnRH secretion. We will then discuss the interactions of these three signaling systems and current controversies, including the possible role of KNDy neurons in the positive feedback of ovarian steroids.

4. The KNDy Signaling Systems

4.1. Kisspeptin

Since the discovery of *Kiss1r* mutations as a cause of hypogonadotropic hypogonadism, considerable evidence has surfaced to support the notion that kisspeptin directly activates GnRH neurons and has little effect at the pituitary. Kisspeptin administration induces Fos expression (marker of neuronal activation) in GnRH neurons [69]. In rats, exogenous administration of kisspeptin potently stimulates FSH [70] and LH [71] secretion. This has also been shown to be the case in mice, sheep [72], goats [73], monkeys [74] and humans [75]. These stimulatory effects are abolished by pretreatment with GnRH antagonists [76]. Kisspeptin also stimulates the release of GnRH *in situ* in rodent hypothalamic explants [77] and induces prolonged trains of action potentials

in GnRH neurons [78–82]. Pulsatile application of kisspeptin induces synchronous *GNRH1* gene transcription and episodic GnRH release in organotypic murine brain slice cultures [83]. In addition, kisspeptin neurons project to and synapse upon GnRH perikarya [84–86] and axon terminals [87,88]. Critically, it has been shown that GnRH neurons express Kiss1r [63,69,72,80], the activation of which is known to increase $[Ca^{2+}]_i$ and inhibit inward rectifying K^+ currents to prolong neuronal activation [89].

Most GnRH neurons lack steroid receptor expression [90]. Prior to the discovery of kisspeptin as a modulator of HPG axis function, the neural systems mediating the feedback actions of gonadal steroids were controversial. The extensive evidence that kisspeptin fibers innervate GnRH perikarya, processes and terminals [65,78,84–88,91,92], and that most kisspeptin neurons express E_2 receptors [93–96] suggests that kisspeptin neurons mediate the feedback effects E_2 on GnRH neurosecretion. Moreover, most current models propose that the ARC population is predominantly associated with negative effects of E_2 on pulsatile gonadotropin secretion, while the rostral kisspeptin neurons are implicated in the positive E_2 feedback that culminates in surge release of GnRH, LH and FSH [97].

Central administration of a kisspeptin bolus seems to stimulate LH secretion by triggering mass release of GnRH from terminals in the ME, but does not affect the electrical activity of KNDy neurons [49,98]. Thus, if kisspeptin is involved in GnRH pulse generation, its release must be pulsatile in order to prevent the desensitization of Kiss1r [99]. However, studies testing this hypothesis have yielded contradictory evidence. Kisspeptin secretion was confirmed to be pulsatile in pubertal female macaques using push-pull perfusion of the ME, and moreover the occurrence of kisspeptin pulses closely correlates with pulses of GnRH [100]. Concomitantly, antagonists of Kiss1r are only able to affect GnRH pulse generator frequency when administered directly into the ARC-ME in either an acute or a pulsatile fashion, with sustained infusions resulting in the abolition of LHpulsatility [68,82,91]. Thus, kisspeptin's role in GnRH pulse generation is arguably to act as a mediator of upstream mechanisms triggering its release (e.g., NKB), and is perhaps limited to evoking robust discharges of GnRH into the portal vasculature from nerve endings in the ME. However, evidence contrary to this notion suggests that central infusion of kisspeptin increases LH pulse frequency in luteal phase ewes [101] and that knockdown of *Kiss1* expression in the rat ARC results in a decrease in LH pulse frequency and disruption of ovarian cyclicity [102,103]. Moreover, pulsatile (as well as surge) secretion of LH was altogether absent in *Kiss1*-knockout rats [104], highlighting the critical importance of this neuropeptide as a component of the GnRH pulse generator.

In man, administration of an iv bolus of kisspeptin results in a single LH pulse that is followed by an endogenously-generated pulse at an interval similar in duration to that of inter-pulse intervals observed before treatment, irrespective of when the kisspeptin-induced pulse occurred with respect to the previous endogenous pulse [105]. Indeed, no evidence of Kiss1r desensitization was observed in men receiving kisspeptin infusions for over 22 hours, and in fact the treatment did increase LH pulse

frequency in these subjects [106]. However, kisspeptin's effect on LH pulse frequency appears to be unique to human males [107], in which the notion of KNDy peptide coexpression has been challenged [108]. Notwithstanding, continuous kisspeptin infusion was able to restore pulsatile LH release in patients (three male and one female) with hypogonadotropic hypogonadism due to mutations causative of impaired NKB signaling [109]. Kisspeptin and its receptor are therefore prime therapeutic targets in reproductive disorders.

In species in which exogenous kisspeptin alters the frequency of the GnRH/LH pulse by means other than merely inserting a single pulse between naturally occurring pulses (namely rat, ewe and man) kisspeptin signaling is considered to affect pulse generation mechanisms. At this time there is one report of Kiss1r in non-KNDy ARC neurons, but not in KNDy neurons in sheep [110]. Until Kiss1r expression by KNDy neurons is interrogated in other species, it will remain unknown whether this effect is direct (auto-stimulatory) or indirect. The latter could occur via adjacent ARC neurons that are kisspeptin-sensitive and have reciprocal connections with KNDy neurons, such as proopiomelanocortin (POMC) [110] and/or tuberoinfundibular dopamine (TIDA) [111] neurons. In summary, kisspeptin signaling plays a critical role in the direct stimulation of pulsatile GnRH release, however whether kisspeptin just serves as the effector of upstream oscillatory mechanisms or also has a modulatory role in such pacemaking activity remains to be elucidated.

4.2. Neurokinin B

The discovery that mutations in genes encoding the neuropeptide neurokinin B (NKB), *TAC3*, or its cognate receptor (NK3R), *TACR3*, can also lead to hypogonadotropic hypogonadism [112,113] has stimulated a substantial research effort addressing NKB regulation of the HPG axis. Moreover, NKB has been found to be coexpressed within KNDy neurons in the ARC, but not in rostral kisspeptin neurons [64,66,114]. In sheep, NK3R mRNA is expressed by most ARC kisspeptin neurons, though GnRH neurons seem to lack NK3R expression [115]. Indeed, central administration of senktide, a synthetic NK3R agonist, increases Fos expression in KNDy neurons [116–118] and stimulates their firing activity but does not alter GnRH neuron firing when bath-applied to acute brain slices from male mice [119,120]. Like kisspeptin, NKB expression, is also subject to the feedback effects of gonadal steroids, as is the case for NK3R [121,122]. Although there is substantial evidence to suggest that NKB signaling has a stimulatory effect on GnRH/LH secretion, which would be expected given the subfertile phenotype of *TAC3* and *TAC3R* mutants, some controversies have recently come to light.

In female rodents, E_2 levels appear to modulate the effect of NKB signaling on pulsatile LH secretion: NK3R agonists induce the release of LH in the presence of high E_2 levels, but suppresses LH pulses in OVX animals with or without replacement of low levels of E_2 (recently reviewed in [123]). Although such effects in other species have not yet been formally reported, there is anecdotal evidence that NK3R activation decreased LH levels in the OVX monkey (Terasawa et al,

personal communication) and we have occasionally observed subtle inhibitory effects on LH secretion in some OVX ewes [124]. Conversely, central administration of the NK3R agonist senktide in OVX goats immediately increased MUA volley frequency, but prolonged the interval between the last NKB-induced volley and the next spontaneous volley, with an overall increase in the duration of the LH pulse interval and a net decrease in LH levels [125]. Moreover, the response of these electrophysiological and endocrine correlates of the GnRH pulse generator to NKB administration was not altered by E_2 or P_4 replacement [125]. Coexpression of estrogen receptor alpha (ERα) within KNDy neurons [96,114] suggests a mechanism for the negative feedback effects of E_2 on NKB/NK3R signaling, but does not explain the disparity of conclusions that may be drawn from different reports on the effects of NKB/NK3R signaling on the HPG axis.

The involvement of NKB signaling in the modulation of the GnRH pulse generator is evident from studies reporting altered frequency of hormonal (i.e., LH) or electrophysiological (i.e., MUA) episodes in response to NK3R activation or blockade with the NK3R antagonist SB222200. For instance, there is an increase or an inhibition of LH pulse frequency in ewes fitted with senktide or SB222200-filled microimplants, respectively, placed in the ARC [91]. Further, MUA volley frequency is increased in goats following intracerebroventricular injection of NKB [126] or peripheral infusion of senktide [127]. Evidence for the retardation of the GnRH pulse generator in pubertal female rats administered chronically with SB222200 and the resulting delay in pubertal onset [128], confirms the necessity of phasic NK3R activation in the ARC for physiologic GnRH pulsatility. Thus NKB of KNDy origin is thought to synchronize the activity of the KNDy network and drive pulsatile release of kisspeptin into the ME to generate GnRH pulses [78]. The intimate functional relationship between the NKB/NK3R and kisspeptin/Kiss1r signaling systems is discussed in more detail below.

4.3. Dynorphin A

Dynorphin A (Dyn) is another neuropeptide that is coexpressed in KNDy neurons [66,114,129]. Dyn is an endogenous opioid peptide (EOP) that preferentially signals through the kappa-opioid receptor (KOR) [130]. The effects of Dyn or its analogues on LH secretion have consistently been reported to be inhibitory in a range of species, [125,131,132], while administration of the KOR antagonist, norbinaltorphimine (nor-BNI), has been confirmed to stimulate the release of gonadotropins in animal models in which endogenous Dyn tone is elevated [91,125,133,134]. Since KNDy neurons have been shown to express ERα [114,135] and there is some evidence that they mediate E_2 negative feedback in rats [136], it was expected that Dyn/KOR signaling would too be stimulated by circulating E_2. Indeed, OVX decreases Dyn mRNA expression in the ovine ARC [137]. Surprisingly, Dyn expression in the murine ARC has been shown to be inhibited by E_2 [122,138] despite its suppressive effect on pulsatile LH secretion. Also at odds with the predominating literature that implicates an endogenous Dyn tone in the inhibition of gonadotropin

secretion are findings suggesting that Dyn/KOR signaling is necessary for the post-ovariectomy increase in LH secretion in mice [122] and that nor-BNI pretreatment attenuates the stimulatory LH response to senktide in diestrous rats [139].

Since it was recently shown that KNDy neurons express KOR in mouse [122] and sheep [140], reported changes in the frequency of LH pulses or associated electrophysiological correlates following experimental modulation of Dyn/KOR signaling in the ARC can be interpreted as demonstration of the importance of a Dyn tone local to the KNDy network in GnRH pulse generation. Direct intra-ARC administration of a Dyn analogue decreases LH pulse frequency in E_2-replaced OVX rats [132] and that of nor-BNI increases LH pulse frequency in luteal phase [133] or OVX [91] ewes. Further, intracerebroventricular administration of nor-BNI decreases the interval between MUA volleys and LH pulses in the OVX goat [125] and sustained peripheral infusion of this antagonist increases LH pulse frequency and, in doing so, advances pubertal development in female rats [134]. In mice, Dyn attenuates the firing frequency of KNDy neurons [78,120] and inhibits slow action potentials responsible for synchronous activation of the KNDy network, while nor-BNI potentiates the latter [78]. Collectively, one can infer from these data that Dyn is an integral component of the GnRH pulse generator resident within the bilateral network of KNDy neurons, where its role is the prevention of secretory fatigue and maintenance of pulsatility.

5. Interactions Between the KNDy Signaling Systems

KNDy neurons of the ARC are intricately interconnected [114,125,129,141–143], innervating each other ipsi- and contralaterally [78], and project to the ME, forming close contacts with GnRH axon terminals [141,144] (Figure 1). In rodents, they also project to and synapse with the rostral hypothalamic population of kisspeptin neurons that in turn innervate GnRH perikarya in the POA [78,92], while in sheep they project directly to both POA kisspeptin neurons and GnRH perikarya in the POA and MBH [145] (Figure 1). Although the intracellular dynamics favourable to the release of kisspeptin, NKB and Dyn are currently unknown, optogenetic stimulation of KNDy neurons at 10 Hz for 2 min every 45 min *in vivo* sustained pulsatile LH release in male mice [146]. However, photostimulation of the entire KNDy population at 20 Hz for 10 s is necessary for maximal depolarization of KNDy neurons, which manifests as a slow excitatory post-synaptic potential (EPSP) in acute hypothalamic slices from OVX mice [78]. The slow EPSPs are dependent on the synaptic release of NKB evoked by action potentials arising from adjacent KNDy neurons—a mechanism by which the activity of the entire KNDy network is apparently synchronized [78]. It also appears that the summation of slow EPSPs arising at multiple KNDy neurons simultaneously translates literally into MUA volleys recorded in the ARC. Contralateral projections between KNDy neurons ensure functional synchrony between hemispheres [78,126].

An interaction between NKB and kisspeptin signaling has been demonstrated by inference from evidence of modification of the effects of one by the other in many species. The pulses of LH

induced by central administration of senktide in prepubertal female rats are blocked by pretreatment with a Kiss1r antagonist [147]. Desensitization of Kiss1r by sustained infusion of kisspeptin also blocks senktide-induced LH pulses in agonadal juvenile macaques [148]. Moreover, LH responses to senktide are absent in Kiss1r-knockout mice [149]. Together these data suggest that NKB signals upstream of kisspeptin to stimulate GnRH pulses. Compelling evidence has recently come to light that signaling through NK3R expressed by KNDy neurons is central to the bilateral synchronization of EPSPs in this population, which elicit pulsatile release of kisspeptin into the ME to drive GnRH neurosecretion [78]. Kisspeptin, in turn, does not affect *per se*, but rather partially occludes the suppressive effect of senktide on LH pulses in OVX rats [132]. However, peripheral coadministration of NKB and kisspeptin yielded an attenuated LH response in men versus kisspeptin alone [150], indicating an inhibitory effect of NK3R activation on kisspeptin signaling, perhaps within the ME or pituitary.

The inhibitory effect of senktide on pulsatile LH secretion evident in OVX rats regardless of steroid replacement, as described earlier (see section on NKB), is dependent on Dyn signaling in the ARC [132]. Agonists of KOR have been shown to dampen the activity of KNDy neurons stimulated by senktide application *in vitro* [120], although the relevance of this finding to pulsatile GnRH/LH secretion remains elusive. It is hypothesized that Dyn is released in response to NKB-ergic autostimulation of KNDy neurons; in animals with high baseline LH levels a short-loop negative feedback mechanism (e.g. GnRH, or a co-released transmitter, signaling at the level of the KNDy neuron) ensures that kisspeptin release is inhibited so that NKB stimulation of KNDy neurons inhibits LH secretion [123]. Under conditions where NKB plays a stimulatory role, corelease of Dyn is evident due to a robust potentiating effect of nor-BNI treatment on the NK3R-dependent slow EPSPs that synchronize the KNDy network [78]. Moreover, pretreatment with nor-BNI enhances the stimulatory effect of senktide on LH secretion in male rats [139]. It is therefore postulated that KNB activation of KNDy NK3R induces Dyn release, which results in the prolonged suppression of the KNDy network through presynaptic inhibition of NKB secretion [78].

In summary, the current model proposed to incorporate the roles of KNDy signaling systems with regards to GnRH pulse generator function postulates that NKB/NK3R signaling, functioning via an auto-stimulatory feedback loop, is responsible for the synchronization of the activity of KNDy neurons, instigating kisspeptin release at the level of the ME, which directly elicits GnRH pulses. Meanwhile Dyn/KOR signaling is postulated to terminate each NKB/kisspeptin-induced GnRH pulse [122,151] by suppressing NKB release from KNDy neurons [78]. While this working model is perhaps an oversimplification of the true mechanism of GnRH pulse generation since it does not reflect the various conflicting data outlined above, it does nevertheless serve as a useful standard for the reconciliation of the complex interactions between neuropeptide signaling systems in the ARC and the wider hypothalamus.

6. Current Controversies

While there is strong evidence for the postulated roles for kisspeptin, NKB, and Dyn in the generation of GnRH pulses, several unresolved issues remain. As discussed above, one of these is whether kisspeptin acts within the ARC to modulate activity of the KNDy neural network, and if so, whether this reflects an action on KNDy neurons or other neural systems within this nucleus. A second aspect of this hypothesis currently under investigation is the cellular mechanism responsible for the time delay between pulse activation by NKB and pulse termination by Dyn. Opioid inhibition of GnRH release probably kicks in within a couple of minutes of the onset of each pulse, based on the effects of the non-selective opioid receptor antagonist, naloxone, on GnRH pulse shape in sheep [152], so that three possibilities can be envisioned: (1) a lag between the release of NKB and Dyn, (2) signaling mechanisms within the KNDy neuron that are faster for NKB than Dyn, or (3) receptor turnover. A recent report that KNDy peptides are segregated in separate vesicles [153] could hint at independent release mechanisms [154] that would allow for a delay in release. There is currently no data that addresses the second possibility, but considerable information on receptor turnover in other systems is available. Both NK3R and KOR are G protein-coupled receptors, which are known to internalize shortly after ligand binding [155,156], so internalization of NK3R could account for the transition from NKB stimulation to Dyn inhibition. Similarly, internalization of KOR could be an important step in activity-dependent resetting of the KNDy network, but there is also another intriguing example of KOR turnover [157]. In magnocellular neurons containing arginine vasopressin (AVP), Dyn is colocalized with AVP in secretory vesicles [158,159] and KOR is expressed within the membrane of these vesicles [160]. Thus when AVP and Dyn are released during a burst of action potentials, the fusion of the vesicle membrane effectively inserts KOR into the plasma membrane [160], which allows Dyn to hyperpolarize the pre-synaptic neuron and terminate the action potential burst [161,162]. By analogy, translocation of KOR from the membrane of NKB secretory vesicles to the plasmalemma in KNDy neurons would be an elegant mechanism for the autocrine termination of each pulse shortly following initiation by NKB release. This hypothesis is yet to be tested, but we have preliminary evidence for the intracellular translocation of KOR in KNDy neurons two minutes after the start of a GnRH pulse [163]. The number of internalized particles is greater at pulse termination 10 minutes following pulse initiation [163], suggesting continued Dyn release as a pulse progresses.

A second current controversy is the role of KNDy neurons in the positive and negative feedback actions of ovarian steroids. As noted above, early kisspeptin models proposed that the ARC population mediates negative feedback, while the more rostral population responds to increasing E_2 and drives the preovulatory GnRH surge [97,164]. The role of KNDy neurons in negative feedback was initially supported by correlative data (i.e., kisspeptin expression inhibited by E_2), but more recent data in rodents has questioned this hypothesis. Thus, at this time there are data that support this hypothesis [165,166] as well as studies that do not [167–169]. In sheep, on the other hand, there is strong evidence that Dyn release from KNDy neurons mediates P_4 inhibition of GnRH/LH pulse

frequency, while inhibition of kisspeptin (and possibly NKB) by E_2 is responsible for inhibition of pulse amplitude [170].

There is still general agreement that the rostral kisspeptin population is largely dedicated to positive feedback and several lines of evidence [62,78,103,171,172] support this, including: (1) this population is sexually dimorphic in most species, with more kisspeptin neurons in females than in males; (2) E_2 stimulates kisspeptin expression in this population, and (3) the expression of Fos in these neurons increases dramatically at the time of the LH surge. KNDy neurons were first proposed to mediate E_2 positive feedback in sheep because this population is sexually dimorphic in this species [65,135]. Several subsequent studies, using Fos expression as an index of neural activity, have implicated these neurons in driving GnRH secretion during the LH surge [63,117,145,173,174], although one study [175] did not reach the same conclusion, possibly for technical reasons [145]. Interestingly, Fos expression also increased in KNDy neurons during the late follicular phase, relative to the luteal phase [63], which is consistent with the notion that the ARC is the site of E_2 positive feedback in the ewe [176]. Although an early study reported a similar increase in ARC kisspeptin/Fos coexpression in rats [177], this was not seen in a subsequent study [171] and the consensus developed that this population was not important for E_2 positive feedback in rodents. However, recent research has supported an important role for kisspeptin from KNDy neurons in LH surge generation in rodents [78,103] and there are now reports that suggest that Dyn from these neurons may inhibit GnRH secretion during the surge [165,178]. In summary, there is strong evidence implicating KNDy neurons in both positive and negative feedback actions of ovarian steroids in sheep, but their role in mice and rats remains controversial.

7. Concluding Remarks

The GnRH pulse generator has been studied through measurement of electrophysiological activity of individual neuronal groups and analysis of hormonal profiles. Recent technological advances and the advent of the transgenic approach have permitted the exquisite targeting of treatments and labels to various components of the HPG axis, and have been instrumental in the characterization of KNDy neuron interplay with the GnRH network (Figure 1). The activity of the KNDy-GnRH axis may play a role in the coordination and amplification of responses to internal signals, such as those relayed by gonadal steroids [151], as well as in the mediation of the effects of external stimuli, including stress [179], metabolic status [180] and photoperiod [181] on the functions of the HPG axis. Because all of these factors have the potential to dramatically influence the parameters of pulsatile gonadotropin release, it is important to thoroughly elucidate the mechanisms underlying GnRH pulse generator activity in a range of experimental species, and to appreciate the fragile nature of this elusive construct.

Conflict of Interest

The authors declare no conflict of interest.

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