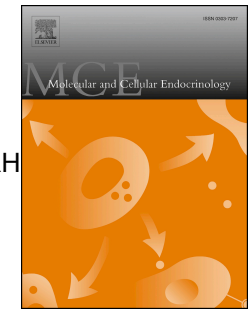

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The KiNG of reproduction: kisspeptin/ nNOS interactions shaping hypothalamic GnRH release

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Abstract

Gonadotropin-releasing hormone (GnRH) is the master regulator of the hypothalamic-pituitary-gonadal (HPG) axis, and therefore of fertility and reproduction. The release pattern of GnRH by the hypothalamus includes both pulses and surges. However, despite a considerable body of evidence in support of a determinant role for kisspeptin, the mechanisms regulating GnRH pulse and surge remain a topic of debate. In this review we challenge the view of kisspeptin as an absolute “monarch”, and instead present the idea of a Kisspeptin-nNOS-GnRH or “KiNG” network that is responsible for generating the “GnRH pulse” and “GnRH surge”. In particular, the neuromodulator nitric oxide (NO) has opposite effects to kisspeptin on GnRH secretion in many respects, acting as the Yin to kisspeptin’s Yang and creating a dynamic system in which kisspeptin provides the “ON” signal, promoting GnRH release, while NO mediates the “OFF” signal, acting as a tonic brake on GnRH secretion. This interplay between an activator and an inhibitor, which is in turn fine-tuned by the gonadal steroid environment, thus leads to the generation of GnRH pulses and surges and is crucial for the proper development and function of the reproductive axis.

Keywords

Nitric oxide, Kisspeptin, Estrogen, GnRH pulse, GnRH surge

1 Introduction

ὁρμῶν (hormôn), “to set in motion, to excite, to stimulate”. Since its introduction by Ernest Starling in 1905 (Starling, 1905), this Greek word has been used to describe the chemical messengers used as a means of communication between different organs in an animal. When the term was introduced, practically nothing was known about the nature or the action of these messengers, which were believed to be produced by only a few specialized organs of the endocrine system (i.e. the glands). We have come a long way since then, with many conceptual changes occurring over the years, the most important of which is possibly the acknowledgement that the nervous and endocrine systems work together to transmit physiological information. The discipline of “Neuroendocrinology” was launched by Geoffrey Harris with his publication in 1955, which not only provided the first proof that the endocrine system could be controlled by the central nervous system (CNS), but also laid the foundations for the notion of the hypothalamic-pituitary-gonadal axis (HPG) (Harris, 1955). In the past two decades, we have come to acknowledge that the field of Neuroendocrinology extends far beyond the traditional neuron-endocrine pathways to encompass the production of hormones by non-traditional cells and tissues, with new and often non-catalytic roles in the regulation of an organism’s development, physiological homeostasis, reproductive capacity and behavior.

The three components of the HPG axis – the hypothalamus, pituitary gland and gonads (i.e. the testes and ovaries) – closely interact and depend on each other to allow the complex dialogue between the CNS and the periphery that is indispensable for reproductive function. The hypothalamus is undeniably the single most important brain region integrating vegetative and endocrine signals, and controls diverse processes including cardiovascular function, sleep, metabolism, stress, thermoregulation, water and electrolyte balance, growth and reproduction. Within the hypothalamus, specialized neuronal populations sense moment-to-moment changes in circulating levels of hormones and nutrients, to regulate the neuroendocrine function (Elmquist et al., 2005). Among these hypothalamic neuronal populations are the neurons producing gonadotropin-releasing hormone (GnRH), the main orchestrators of reproductive function, which act as integrators of various signals coming from both the central and the peripheral nervous system.

In spite of their crucial role, GnRH neurons are an extremely small population of cells across mammalian species, counting only 1,000−3,000 neurons in rodent brain. The GnRH neuronal soma are primarily distributed in the preoptic hypothalamic area (POA) extending their nerve terminals to the pericapillary space of the median eminence (ME), located in the more mediobasal area of the hypothalamus (MBH), releasing the GnRH decapeptide in an episodic manner in both sexes (Sarkar et al., 1976; Moenter et al., 1991; Sarkar & Minami,

1995, Terasawa et al, 1999). Indeed, both immortalized GnRH-secreting GT1 cells and primary GnRH neurons release GnRH in a pulsatile manner, at species-specific intervals (for review see Terasawa, 2019). GnRH is then carried through the pituitary portal circulation for delivery to the anterior pituitary, where it stimulates gonadotropes to synthesize and secrete the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH). These gonadotropins then act on the gonads (i.e. the testes and ovaries) to promote gonadal development and the secretion of sex steroids, which in turn provide positive or negative feedback back to the brain to regulate GnRH release (Prevot, 2015). The pulsatile pattern of GnRH secretion is reflected in the pulsatile secretion of LH during the negative feedback action of sex steroids, while GnRH surge, taking place during the positive feedback action of sex steroids, results in a crucial peak of LH release, triggering ovulation in females (Nett et al., 1974).

Thus, the correct release of LH and FSH depends on the regulation of the frequency and timing of GnRH secretion by sex steroids as well as other neuronal and non-neuronal factors. In turn, the proper development of GnRH neurons, GnRH expression and GnRH signaling are all essential for the normal functioning of the mammalian HPG axis (Cattanach et al., 1977; Mason et al., 1986; Schwanzel-Fukuda et al., 1989). Justified by the authority of the GnRH system over the regulation of key physiological events, the network surrounding GnRH neurons, ensuring the controlled and timely regulation of their response is complex and multidimensional. To date, kisspeptin neurons have so far been considered the master excitatory driving force behind GnRH/LH release during both positive and negative feedback phases (Navarro et al., 2009; Pielecka-Fortuna et al., 2010; Clarkson et al., 2017).

In this review, based primarily on research carried out in rodents, we challenge the view of kisspeptin neurons as the sole regulators or supreme “monarchs” of the GnRH network, controlling both GnRH pulse and surge generation. We also explore the implication of the much overlooked population of neuronal nitric oxide synthase (nNOS) neurons, producing the diffusible messenger nitric oxide (NO), in the control of the GnRH system. Finally, we will discuss how the tripartite Kisspeptin, nNOS, GnRH (KiNG) network could be essential for the regulation of LH pulsatility and LH surge.

2 Milestones during the developmental maturation of the GnRH network in the mouse.

GnRH neurons originate from stem cells of the olfactory placode. Around embryonic (E) day 11.5 in mice, these neurons embark on their migratory path from the nose, entering the forebrain following a series of guidance cues (for review see Wray et al., 2010), and in close association with the vomeronasal nerve fibers. By E16, GnRH neurons enter the

forebrain (Schwanzel-Fukuda et al., 1989). The HPG axis is believed to be already somewhat functional during embryonic development (Aubert et al. 1985; Pointis et al., 1980); along with the establishment of the first GnRH projections the fetal pituitary gland becomes GnRH responsive, while fetal testes have been shown to be LH-responsive, secreting steroid hormones (Pointis & Mahoudeau, 1979). At birth, i.e. postnatal day (P) 0, GnRH neurons have reached their final destination in the brain, principally in the hypothalamus, established neuronal afferent connections, extended their processes to the ME and are able to secrete the GnRH peptide into the pituitary portal circulation (Messina & Giacobini, 2013). GnRH starts to be released during the early stages of development (Terasawa & Fernandez, 2001), and even though any alterations during their embryonic development can affect postnatal life (Vanacker et al., 2020), the connectivity, biosynthetic profile and overall activity of GnRH neurons are relatively immature prior to puberty.

Postnatal development in mice can be divided into four stages with regard to GnRH secretion: neonatal (P0-P7), infantile (P8-P21), juvenile (P22-P30) and peripubertal (Prevot, 2015). GnRH levels increase gradually during the neonatal period and remain fairly constant during the first few days of the infantile period. The first postnatal centrally-driven and gonad-independent activation of the HPG axis occurs around the first few months of life in humans and during the second week of life in mice (Kuiri-Hänninen et al., 2014; Prevot, 2015). This quite recently described developmental period has been named minipuberty. During minipuberty, gonadotropin levels (i.e. LH and FSH) rise, setting in motion the growth of the first ovarian follicles and estrogen production in females and the development of the testes in males (François et al., 2017; Kuiri-Hänninen et al., 2014; Prevot, 2015). Later during development, a combination trans-synaptic and non-trans-synaptic inputs results in the establishment of GnRH release from the hypothalamus, indispensable for the initiation of puberty (for review see Ojeda & Skinner, 2006; Terasawa & Fernandez, 2001; Plant & Witchel, 2006; Prevot, 2015). Although gonadal steroid hormone production gradually increases over minipuberty, estrogen remains unable to act upon the hypothalamus because of the presence of high levels of the estradiol-binding α-fetoprotein (AFP) in the circulation (Raynaud et al., 1971). Hence, the minipubertal high levels of FSH secretion accompanied by low levels of LH release are the result of the low-frequency GnRH secretion along with the absence of an operational negative feedback mechanism (Raynaud et al., 1971). At the end of the infantile period (i.e. the beginning of the juvenile stage), the negative feedback of ovarian estrogen is fully operational, resulting in a decrease of FSH to nadir values, while plasma LH levels remain low. By the end of the juvenile stage, GnRH neurons will be primed to respond to both low and preovulatory estrogen levels (Prevot, 2015).

Throughout postnatal development, the dendrites of GnRH neurons undergo morphological remodeling, critical for their network integration and the establishment of the

postpubertal GnRH neurosecretory pattern (Cottrell et al., 2006; Ybarra et al., 2011). These intrinsic changes in GnRH neurons are accompanied by a reorganization of the afferent hypothalamic network shaping reproductive capacity (Figure1). For instance, neuronal populations located in the region of the anteroventral periventricular nucleus (AVPV), believed to be responsible for mediating the positive feedback action of gonadal steroids, have already established their projections to GnRH neurons in the median preoptic nucleus (MePO) by birth (Polston & Simerly, 2009). In contrast, axonal projections coming from the arcuate nucleus of the hypothalamus (ARH), a region believed to contain neuronal populations that mediate the negative feedback action of gonadal steroids (Wintermantel et al., 2006), have only been observed to reach GnRH neurons around minipuberty, although they expand throughout the infantile period (Bouret et al., 2004; Caron et al., 2012) (Figure1). How differences in the timing of afferent synaptic connectivity affect the maturation of the GnRH system remains rather obscure, as does the identity of the key neuronal populations involved in this process. What is beyond doubt is that any dissonance in the factors responsible for regulating the complex pattern of GnRH secretion, be they genetic, nutritional, environmental or even socioeconomic, jeopardize reproductive capacity by perturbing the timing of key developmental events (Livadas & Chrousos, 2019).

2.1 Kisspeptin: the "Yang" of the GnRH network.

The *Kiss1* gene was initially identified as metastasis suppressor and kisspeptin has been originally isolated from human placenta for its ability to inhibit tumor metastasis and progression (Kotani et al., 2001; Ohtaki et al., 2001). However, the identification of hypogonadotropic hypogonadism and infertility in both humans and mice harboring deleterious mutations in the genes encoding kisspeptin or its receptor, GPR54, strongly pointed towards a key role for kisspeptin signaling in reproductive function (de Roux et al., 2003; Seminara et al., 2003; Topaloglu et al., 2012). In accordance with this role, GPR54 is known to be expressed by GnRH neurons (Irwig et al., 2004; Messager et al., 2005), a finding that has contributed to the recognition of the central role of kisspeptin-GPR54 signaling in the neuroendocrine system (for review see Tena-Sempere, 2010).

The *Kiss1* gene encodes a 121 amino-acid protein that is cleaved into a number of smaller kisspeptin peptides. The longest of these is 54 amino acids in length (Kp54), but shorter kisspeptins (Kp13, Kp14) that may represent degradation products still retain full biological activity, as does a synthetic peptide of only 10 amino acids (Kp10) (Kotani et al., 2001; Ohtaki et al., 2001). Kisspeptin, through GPR54 signaling, induces G-protein (Gαq/11)-mediated cascades, such as the activation of phospholipase C, leading to the intracellular accumulation of inositol triphosphate (IP3) and diacylglycerol (DAG). The increase in

intracellular Ca^{2+} and DAG activates protein kinase C and initiates pathways related to mitogen-activated protein kinases (MAPKs), especially ERK1/2 and p38, and phosphatidylinositol-3-kinase (PI3K)/Akt. DAG also stimulates GnRH depolarization by the activation of a nonselective cation channel (TRPC) and inhibition of an inwardly rectifying potassium channel (K_{ir}) (for extensive review see Castaño et al., 2009). Thus, kisspeptin acts directly on GnRH neurons to induce a sustained depolarization event and action potential firing (reviewed in d'Anglemont de Tassigny, & Colledge, 2010).

Only two populations of *Kiss1*-expressing cells have been identified in the hypothalamus of virtually every mammalian species examined so far, and are located specifically in areas key to steroid hormone feedback control: the POA and the MBH. In rodents, the kisspeptin neuron population of the POA is located within the rostral periventricular area of the third ventricle (RP3V), the area comprising the AVPV and the adjacent preoptic periventricular nucleus, while the MBH population is found throughout the ARH (Kumar et al., 2015).

In rodents, kisspeptin immunoreactivity in the AVPV is observed for the first time during the infantile period, around P10, and undergoes a substantial and steady increase over the following days until the end of the juvenile period (Semaan & Kauffman, 2010), while the expression of *Kiss1* mRNA increases dramatically during the transition from juvenile to adult life (Han et al., 2005). Tracing studies have revealed that AVPV kisspeptin neurons directly project to the somata of GnRH neurons in the organum vasculosum laminae terminalis (OV) and MePO (Yip et al., 2015), which express the kisspeptin receptor GPR54 (Irwig et al., 2004). The apposition of kisspeptin-immunoreactive fibers from the AVPV onto GnRH neurons in the OV/ MePO is actually undetectable in female mice before P25 (Clarkson & Herbison, 2006). The majority of AVPV kisspeptin neurons are GABAergic, with only 20% showing a glutamatergic phenotype (Cravo et al., 2011).

In contrast to the AVPV kisspeptin population, kisspeptin expression in the ARH is already visible around P3 (Takumi et al., 2011), and increases during postnatal development, reaching levels comparable to that of adults by the end of the infantile stage (Navarro et al., 2012) (Figure1). Kisspeptin neurons of the ARH form a dense network of kisspeptin fiber within the nucleus, also projecting to several other hypothalamic populations including the AVPV kisspeptin neurons (Yeo & Herbison, 2011; Yip et al., 2015). ARH kisspeptin neurons coexpress neurokinin B (NKB) and dynorphin (Dyn), and are thus also known as KNDy neurons (Goodman et al., 2007; Navarro et al., 2009; Cravo et al., 2011). Even though single-labeled kisspeptin and NKB fibers can be seen in the ME of rodents contacting the distal projections of GnRH neurons (i.e. dendrons) axons from KNDy neurons project mainly to one another as well as to the internal zone of the ME (Ciofi et al., 2006; Yip et al., 2015). ARH kisspeptin neurons are mostly glutamatergic, and they use glutamatergic transmission

to increase the activity of neuronal populations important for metabolic circuitry, such as agouti-related peptide (AgRP) and proopiomelanocortin (POMC) neurons (Nestor et al., 2016). In addition, glutamatergic excitation is used by ARH kisspeptin neurons as means of communication with the AVPV kisspeptin population, likely being a critical mechanism for the preovulatory GnRH/LH surge (Pielecka-Fortuna et al., 2010; Qiu et al., 2016).

2.2 NO: the "Yin" of the GnRH network.

The biologically active gaseous compound NO is a simple, membrane-permeable molecule with unique chemistry. The presence of an unpaired electron renders it highly reactive with other free radical species but extremely unreactive to other biological molecules. It is formed by the enzymatic oxidation of L-arginine to L-citrulline in the presence of oxygen and NADPH, with flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), heme, thiol, and tetrahydrobiopterin as cofactors (reviewed in Chachlaki & Prevot, 2020). The above reaction involves the electron transfer between the oxygenase and reductase domains of the nitric oxide synthase (NOS), the enzyme responsible for the conversion of L-arginine to NO, and requires the dimerization of the NOS protein (Chachlaki & Prevot, 2020). NOS exists in three different isoforms: inducible NOS (iNOS or NOS2), endothelial NOS (eNOS or NOS3) and neuronal NOS (nNOS or NOS1); the latter is responsible for the production of NO in neuronal cells (Bredt et al., 1991). nNOS activation and the subsequent production of NO are dependent on Ca^{2+} influx through open NMDA receptor (NMDAR) channels. The rise in intracellular Ca^{2+} results in its binding to calmodulin, creating a Ca^{2+}/calmodulin complex, leading to nNOS becoming catalytically active (Garthwaite et al., 1988). The phosphorylation of the NMDAR-tethered nNOS (by the protein kinase Akt) at serine-1412 then leads to a rapid enhancement of nNOS activity and the consequent production of NO by the conversion of L-arginine to L-citrulline (Adak et al., 2001) (Figure 2).

The biological and physiological effects of NO are influenced by the ambient concentration of NO, i.e. the balance between its rate of synthesis and its rate of inactivation, as well as the distance between its site of production and its potential targets (Garthwaite, 2016; Garthwaite, 2019). According to the NO diffusion coefficients (corresponding to an inactivation rate constant of 100–150 s^{-1}, equivalent to a half-life of 5–7 ms), NO is produced as a pulse, peaking at a rate of 40 molecules s^{-1}, with half the NO flowing each side of the emission zone through all available surfaces, in a radius of about 0.2 μm (Bellefontaine et al., 2014; Garthwaite, 2016). This suitably high rate of NO inactivation endows constituent cells with a modality switch, permitting local signaling when their activity is sparse or uncoordinated, and a volume signal when there is synchronous population activity, thus conferring good discrimination and dynamics. Therefore, NO can modulate neuronal activity

by acting both at the level of an individual synapse (Batchelor et al., 2010; Garthwaite, 2010), as well as an intracellular messenger (Zhong et al., 2015). Once NO is released it can freely diffuse across biological membranes, stimulating the production of the second messenger cGMP by binding to soluble guanylate cyclase (sGC), the only known receptor for NO, in target cells (Garthwaite, 2019) (Figure 2). This ability of NO to act as a volume transmitter is what renders it unique among neurotransmitters; its low molecular mass (30 g mol−1) and its rapid rate of aqueous diffusion (~2.5 μm in 1 ms) allow NO from multiple sources to accumulate and diffuse within a certain tissue volume, influencing the activity of populations of cells located at a distance from the source, regardless of synaptic connectivity (Bellefontaine et al., 2014; Garthwaite, 2016).

NO has been evidenced to regulate major aspects of reproductive physiology (reviewed in Bellefontaine et al., 2011); before the discovery of the crucial role of kisspeptin in the control of GnRH release (reviewed in Seminara & Crowley, 2008), *in vitro* and *in vivo* studies had already identified NO as a key molecule for the preovulatory GnRH/LH surge (Moretto et al., 1993; Rettori et al., 1993; Bonavera et al., 1993; Bonavera et al., 1994). The generation of an nNOS knockout mouse model (lacking exon 6 of the *Nos1* gene, which harbors the catalytic domain of nNOS) resulted in hypogonadotropic hypogonadism and infertility accompanied by altered GnRH content and abnormal circulating levels of gonadotropins, supporting the involvement of neuronal NO in the control of the GnRH axis (Gyurko et al., 2002). Hypothalamic NO has in fact been involved in various functions due to the diverse patterns of expression and the heterogeneity of the neuronal populations expressing nNOS (Chachlaki, Malone et al, 2017; Chachlaki & Prevot, 2020).

Anatomically, nNOS cells are part of the kisspeptin/GnRH network across species (Herbison et al., 1996; Chachlaki, Malone et al., 2017; Bedenbaugh, McCosh et al., 2018; Bedenbaugh, O'Connell et al., 2018). In contrast to the more constrained expression pattern of kisspeptin, neuronal NO has been shown to be produced in several hypothalamic areas and nuclei, including the POA, the MBH, the supraoptic nucleus, the paraventricular nucleus of the hypothalamus, and the ventral premammillary nucleus (Chachlaki, Malone et al, 2017). These neuronal populations can be distinguished not only according to their anatomical organization, but also based on phenotypic characteristics such as their glutamatergic or GABAergic identity, or their ability to interact with other key hypothalamic molecules including estrogen and leptin (Bellefontaine et al., 2014; Chachlaki, Malone et al., 2017).

In mice, glutamatergic nNOS-expressing neurons are particularly abundant in the rostral part of the POA, where they are intermingled with GnRH neurons, most of which also share a glutamatergic phenotype (Chachlaki, Malone et al., 2017). nNOS cells of the POA are found in the vicinity of GnRH dendrites in the OV, while surrounding GnRH neuronal cell bodies in the MePO (Herbison et al., 1996; Clasadonte et al., 2008; Chachlaki, Malone et al.,

2017). At minipuberty, the activation of the GnRH axis (i.e. the peak of FSH) is concomitant with a substantial increase in nNOS enzymatic activity, as demonstrated by the increase in serine-1412 phosphorylation levels of nNOS in the POA (Messina et al., 2016). Hence, while nNOS-immunoreactivity is itself unchanged during this time, NO levels are greatly increased in the POA (Messina et al., 2016; Chachlaki et al., 2017) (Figure1).

In the MBH, large populations of mainly glutamatergic nNOS cells are found in the dorsomedial (DMH) and ventromedial hypothalamus (VMH) while a sparser GABAergic population is seen in the ARH, morphologically associating with GnRH terminals (Chachlaki, Malone et al, 2017). Even though nNOS-immunoreactivity in the POA at P11 already occurs at levels similar to those seen in adult mice, nNOS expression in the ARH is absent at the time of minipuberty (Chachlaki, Malone et al., 2017) (Figure1). This spatiotemporally regulated nNOS expression could in fact be linked to the action of the gonadal steroids, which, as mentioned above, are known to significantly increase during minipuberty (François et al., 2017; Prevot, 2015).

3 The involvement of ovarian steroid hormones in the GnRH pulse and the GnRH surge generators.

Even though the secretory profiles of estrogen and progesterone are poorly characterized in mice, both gonadal steroids exert critical inhibitory and stimulatory actions upon the brain to control GnRH release, shaping the estrous cycle in all female mammals.

It is widely accepted that estrogens play a key role, exerting a negative feedback action on the GnRH system, thus maintaining the constant frequency of the episodic release of GnRH/LH throughout the follicular phase. According to the “estrogen switch” model, depending on the phase of the female estrous cycle, estrogen either has a positive feedback effect, reinforcing GnRH release, or a negative feedback effect, inhibiting this release. With the exception of proestrus, estrogen’s role during the cycle is mostly negative upon the GnRH pulse generator (Moenter et al., 2019; Plant, 2020). This negative feedback of estrogen on the GnRH system maintains the homeostatic control of the GnRH/LH pulsatile release. Oppositely, during proestrus, sustained high concentrations of circulating estrogens produced by mature ovarian follicles enhance the secretion of GnRH. This positive feedback action of estrogens on the GnRH system eventually provokes the GnRH/ LH surge (Knobil, 1980).

Extending this oversimplified “estrogen switch” model, one could imagine that GnRH pulsatility and the GnRH surge are actually maintained by an alteration of stimulatory and inhibitory neuronal inputs in response to fluctuating estrogen levels. Whether estrogen operates this inhibitory-stimulatory switch through a specific afferent neuronal population or

whether it acts conjointly on different components of the GnRH neuronal network remains elusive, but its opposing effects seem to involve distinct hypothalamic regions, as we will detail further down. Estrogen receptor α (ERα) is essential for mammalian physiology and reproduction (Wintermantel et al., 2006). Neuron-specific deletion of ERα from glutamatergic, GABAergic or kisspeptin neurons in the brain have revealed its crucial role in mediating both types of feedback action within the GnRH network (Cheong et al., 2015; Dubois et al., 2015). The fact that GnRH neurons do not themselves express ERα (Watson et al., 1992; Herbison & Theodosis, 1992) suggests that estrogen targets other ERα-expressing cells afferent to GnRH neurons, such as certain populations of kisspeptin or nNOS neurons, in order to convey the hormonal information required for the control of GnRH release.

The action of progesterone is key to the synchronization of follicular development and the maintenance of the correct duration of the luteal phase, although the underlying mechanisms as well as the role of progesterone in shaping the frequency of the pulse remain relatively unknown. Across species, progesterone suppresses estrogen positive feedback and consequently the secretion of GnRH/LH (Levine, 2015; He et al., 2017; Han et al., 2019). GnRH neurons do not express the progesterone receptor (PR) (Le et al., 1997). However, AVPV and ARH kisspeptin neurons have been shown to express PR in both sheep and rodents (Clarkson et al., 2008). Studies in sheep have suggested that progesterone's inhibitory effect stems from its stimulatory action on dynorphin (Dyn) signaling (Goodman et al., 2004), while studies in rats have suggested that progesterone suppresses the GnRH/LH surge by blocking the ability of estradiol to induce the surge (He et al., 2017). It is important to note that most of these studies have examined the effects of exogenously administered progesterone or ovarian progesterone production and its association with PR expression. In the brain, however, rising estradiol levels have been shown to promote the de novo synthesis of progesterone in astrocytes, and this "neuroprogesterone" (neuroP) amplifies the GnRH/LH surge (Micevych et al., 2007; Mittelman-Smith et al., 2018). The mechanism by which neuroP augments GnRH release is dependent upon estradiol-induced kisspeptin expression in the AVPV (for a broader understanding see review by Sinchak et al., 2020). Although nNOS neurons of the POA have been shown to express PR in guinea pigs (Warembourg et al., 1999), very little is known about the effect of neuroP on nNOS neurons or its implications for GnRH release.

3.1 The divergent role of estrogen on the AVPV and ARH kisspeptin populations.

Kisspeptin is commonly thought to play the role of a lone and supreme regulator, a sort of "monarch", in the GnRH network. The majority of kisspeptin neurons in both the AVPV and ARH express ERα (Franceschini et al., 2006; Smith et al., 2005, Smith, Dungan et al.,

2005). However, estrogen action on these two populations diverges, since estrogen upregulates kisspeptin expression in the AVPV, while it downregulates *Kiss1* mRNA levels in the ARH (Smith et al., 2005, Smith, Dungan et al., 2005).

Kisspeptin is known to be a potent stimulator of GnRH neurons, promoting GnRH release via mechanisms enabled by a rise in estrogen levels (Messager et al., 2005; Pielecka-Fortuna et al., 2008). AVPV kisspeptin content is increased by estrogen and the activation of ERα in kisspeptin neurons is required for the positive feedback action of estrogens. Indeed, stimulation of kisspeptin by estrogen in the AVPV of mice in which ERα is specifically deleted in kisspeptin neurons (KERαKO) failed to elicit the GnRH/LH surge (Dubois et al., 2015). More recently, the selective knockdown of ERα in the AVPV kisspeptin population of adult mice has been shown to lead to decreased excitability in kisspeptin cells, blunting the LH surge (Wang et al., 2019). Consequently, as we will discuss in the next section, the AVPV kisspeptin population is postulated to be the main component of the GnRH surge generator.

In contrast to the AVPV kisspeptin population, the expression of Kiss1, Dyn and NKB in the ARH have been shown to be negatively regulated by estrogen levels (for review see Lehman et al., 2010). In fact, KNDy neurons, display “nonclassical bursts”, in a gonadal-dependent manner, with the absence of gonadal steroid feedback resulting in increased excitability and ultimately elevated GnRH/LH secretion (Vanacker et al., 2017). Intriguingly, LH pulsatility seems to be associated with multiunit electrical activity (MUA) in the ARH, supporting the notion that the GnRH pulse generator lies inside the ARH and actually involves the synchronized behavior of KNDy neurons, a hypothesis that we will question in the following sections (Thiéry & Pelletier, 1981; Kawakami et al., 1982; Wilson et al., 1984).

3.2 The impact of estrogen on nNOS enzymatic activity.

Neuroanatomical studies in mice and other species have revealed that in addition to kisspeptin neurons, ERα-expression is extensively present in the nNOS neurons in both the POA (AVPV and OV/MePO) and the ARH (Chachlaki, Malone et al., 2017; Bedenbaugh, O’Connell et al., 2018). NO is believed to regulate GnRH/LH release in a gonadal-dependent manner; In the presence of gonadal steroid positive feedback (i.e. during proestrus) or in absence of gonadal steroid negative feedback (i.e. upon male castration), *in vivo* pharmacological inhibition of central NO production via the intracerebroventricular infusion of N(G)-nitro-L-arginine methyl ester (L-NAME) rapidly suppresses pulsatile LH release (Rettori et al., 1993; Hanchate et al., 2012). Conversely, when negative feedback by gonadal steroid operates (i.e. during diestrus) inhibition of NO release increases LH to levels comparable to those seen in proestrus (Hanchate et al., 2012).

In the OV/MePO, where GnRH neurons reside, ERα expression is almost exclusively present in nNOS cells, highlighting the importance of this discrete neuronal population in sensing gonadal information (Chachlaki, Malone et al., 2017). Indeed, the critical involvement of estrogen in the control of nNOS activity in the POA has been well-documented. Estrogen promotes the coupling of glutamatergic influx with NO production in the ERα-expressing nNOS neurons of the POA, with NO levels being significantly increased in proestrus, concomitant with the preovulatory increase in estrogen levels (d'Anglemont de Tassigny et al., 2007). Specifically, high estrogen levels during proestrus promote the association of nNOS with NMDAR through the scaffolding protein PSD-95, leading to its activatory phosphorylation (d'Anglemont de Tassigny et al., 2007; d'Anglemont de Tassigny et al., 2009; Hanchate et al., 2012; Parkash et al., 2010) and subsequent NO release (Rameau et al., 2007). Conversely, nNOS dephosphorylation in POA protein extracts (induced by a λ-phosphatase treatment) dramatically reduces NO production (Parkash et al., 2010), while the knockdown of PSD-95 disrupts estrous cyclicity (d'Anglemont de Tassigny et al., 2007).

As mentioned above, the ARH is believed to contain the neuronal populations involved in the estrogen negative feedback mechanism and thus LH pulse generation. The expression of ERα from the ARH nNOS neurons suggests that this neuronal population of NO-producing cells is indeed sensitive to alterations in the estrogen levels and could thus potentially be implicated in the network mediating the negative feedback action of estrogen (Chachlaki, Malone et al., 2017). In line with this hypothesis, constitutive basal levels of NO during the negative feedback by gonadal steroid operates (i.e. during diestrus) are thought to impose a tonic brake on LH secretion (Hanchate et al., 2012). NO could exert its restraining effect, believed to be crucial for LH pulsatility, directly on GnRH neurons at the level of the soma (Clasadonte et al., 2008), or at the level of the neuroendocrine terminals of GnRH neurons (Bonavera et al., 1993; Rettori et al., 1993; Bonavera et al., 1994; Prevot et al., 1999; Knauf et al., 2001; de Seranno et al., 2004). In addition, NO's effect could be indirect, by acting trans-synaptically on their afferents (Pielecka- Fortuna et al., 2008; Zhang et al., 2009; Pielecka-Fortuna and Moenter, 2010), or even by a more complex mechanism involving both direct and indirect interactions.

4 The tripartite KiNG network: Could NO be the yin to kisspeptin's yang?

4.1 The mechanism underlying the ability of nNOS cells to promote the synchronized activity of GnRH neurons

Since mammalian GnRH neurons are widely scattered in the POA, one could hypothesize that GnRH pulsatility and GnRH surge are dependent upon a form of oscillatory network activity rather than individual synaptic connectivity. Although this concept remains to

be uncovered *in vivo,* this oscillatory activity may ultimately reflect the wave-like shape of both pulsatile and surge GnRH secretion. Considering that nNOS neurons can communicate with each other via NO volume transmission, NO signaling is an ideal candidate to orchestrate synchronous GnRH release. nNOS neurons are implicated in the modulation of GnRH neuronal excitability and secretion both *in vitro* and *in vivo*. The effect of nNOS neurons on GnRH release is believed to stem from the ability of endogenous NO to act as a synchronizing agent, contributing to the pulsatile secretion of the neuropeptide by bringing GnRH neurons into phase (Moretto et al., 1993; Wetsel, 1995; López et al., 1997). However, it should be remembered that a synchronized increase in neuronal activity could result not only by the stimulation of a large number of neurons (e.g. GnRH neurons) but also by the lifting of a restrictive influence upon them. Accordingly, spontaneous, i.e. unsynchronized, GnRH neuronal firing has been shown to be strongly inhibited by both endogenous and exogenous NO in a sGC/cGMP dependent manner in *ex vivo* electrophysiological studies on GnRH-GFP mouse brain slices (Clasadonte et al., 2008). How does hypothalamic NO sustain these two apparently contradictory actions, mediating a tonic inhibitory effect on GnRH neuronal activity yet promoting GnRH pulsatility and the GnRH surge?

While it is clear that kisspeptin neurons trigger GnRH release, the mechanism allowing GnRH neurons to recover from their refractory period and return to baseline, thus shaping GnRH pulses and surges, is unknown. Successive waves of NO pulses (~10–20 Hz) are believed to be needed to elicit the accumulation of cGMP to an active concentration range at synapses (Garthwaite, 2016). As a matter of fact, computer modeling of NO signaling in the OV/MePO has highlighted the importance of nNOS phosphorylation in the buildup of NO to concentrations sufficient to engage neighboring GnRH neurons, leading to LH release (Bellefontaine et al., 2014; Garthwaite, 2016). Even though NO volume transmission has so far been demonstrated only after stimulation of nNOS neurons of the OV/MePO by leptin (Bellefontaine et al., 2014), we cannot exclude that this mechanism could similarly be used by other neural pathways. Under this premise, the phosphorylation of nNOS enzyme, and as a result NO volume transmission, might be a mechanism used by members of the GnRH neuronal network to control GnRH release. For example, as mentioned above, estrogenic levels largely affect the activity of nNOS neurons, and consequently their action upon the GnRH network (d'Anglemont de Tassigny et al., 2007; d'Anglemont de Tassigny et al., 2009; Hanchate et al., 2012; Parkash et al., 2010). Similarly, AVPV kisspeptin neurons directly innervate nNOS neurons in the POA, which abundantly express GPR54, and kisspeptin can promote nNOS phosphorylation and subsequently NO release (Hanchate et al., 2012). Hence kisspeptin, similarly to leptin, might use the easily diffusible NO as means of communication to the scattered GnRH neurons indirectly, regulating the GnRH release.

Although current data suggest a key role for nNOS phosphorylation and the resulting NO volume transmission in the regulation of the GnRH surge and pulse, the generation of a knock-in mouse model in which nNOS phosphorylation is eliminated by mutating serine-1412 to a non-phosphorylatable alanine ($nNOS^{S1412A}$) contradicts its importance (Guerra et al., 2020). $nNOS^{S1412A}$ homozygous female mice do not have gross differences in reproductive organ histology, gonadotropin or sex steroid levels, or ovarian cyclicity, implying that serine-1412 phosphorylation of nNOS protein may not be involved in the control of the reproductive axis. However, the punctual abolishment of nNOS serine-1412 phosphorylation attenuates NO production (Parkash et al., 2010), and the transitory lack of nNOS serine-1412 phosphorylation impairs the LH surge and estrous cyclicity (d'Anglemont de Tassigny et al., 2007; Hanchate et al., 2012). The potency of NO signaling depends on its ambient concentration rather than individual synaptic connections between nNOS neurons and their target cells (Chachlaki et al., 2017; Garthwaite, 2016). Hence, the contradictory results obtained from the $nNOS^{S1412A}$ mouse model could actually be the effect of a compensatory developmental mechanism being established to ensure that the NO levels necessary for the normal functioning of the GnRH axis are built up through other pathways. Therefore, it remains critical to assess active NO levels, since the adaptation of the NO signaling cascade, e.g. by decreased elimination rates (i.e. increased phosphodiesterase activity), may result in normalizing NO levels to wild-type physiological values, blunting the expected effect of the genetic mutations introduced.

4.2 Interaction of nNOS and kisspeptin neurons as part of the "GnRH pulse" generator

As detailed above, studies over more than a decade have led to the hypothesis that ARH neurons, and specifically its KNDy neuronal population, are responsible for mediating the negative feedback of estrogens, promoting the synchronization of pulsatile GnRH release (for extensive review, see Lehman et al., 2020; Moore et al., 2018). ARH kisspeptin neurons exhibit brief synchronized episodes of calcium activity throughout the female estrous cycle (McQuillan et al., 2019). This oscillatory activity is tightly associated with pulsatile LH secretion (Keen et al. 2008; Clarkson et al., 2017; Kim et al., 2020), while the optogenetic inhibition of this population is sufficient to blunt pulsatile LH secretion in castrated mice (Clarkson et al., 2017). This synchronized calcium oscillation has been suggested to involve NMDA- and $GABA_A$ receptor-dependent signaling in organotypic slice cultures from neonatal mice (Kim et al., 2020). According to the "KNDy model", NKB activates KNDy cells by acting on the neurokinin 3 receptor (NK3R), leading to a brief release of kisspeptin (for about 1 minute) that subsequently stimulates GnRH secretion. In a second step, KNDy neurons also release Dyn, which in turn acts in an autocrine manner on the κ-opioid receptor (KOR) to

arrest kisspeptin release and thus terminate the GnRH pulse (Lehman et al., 2010; Qiu et al., 2016; Voliotis et al., 2019). However, is Dyn really the “OFF” signal for GnRH neurons?

Intriguingly, in the absence of gonadal steroid feedback, alterations in Dyn signaling do not affect LH pulsatility raising the possibility that KNDy neurons do not provide the pulse termination signal in castrated animals, and are not the estrogen action site exerting the negative feedback effect on LH pulses (at least not via the classic Dyn-KOR signaling pathway) (Mostari et al., 2013). The prevailing notion that these KNDy neurons are themselves the GnRH pulse generator falls further short in light of recent data. The optogenetic activation of KNDy neurons in intact mice perfectly mimics the LH pulse profile (i.e. in amplitude and frequency) (Han et al., 2015). However, in gonadectomized mice, i.e. in the absence of the negative feedback mechanism, the repeated optogenetic activation of KNDy neurons exerts, surprisingly, a slow inhibition of LH production (Han et al. 2020). It remains possible that estrogen could shape LH pulsatility by acting through other pathways of the ARH, independent of kisspeptin cells (Franceschini et al., 2006; Smith et al., 2005, Smith, Dungan et al., 2005). Indeed, even though ERα expression in kisspeptin neurons is needed for the downregulation of kisspeptin in the ARH, it is suggested to be dispensable for the actual negative feedback action of estrogen (Dubois et al., 2015). The knockdown of ERα in the ARH kisspeptin population of adult mice results in reduction of the kisspeptin-induced GnRH/LH secretion, i.e. disruption of GnRH neuronal response to kisspeptin, yet has no effect on the firing rate of these cells or the frequency of the LH pulse (Wang et al., 2019). This apparent redundancy of ERα expression in ARH kisspeptin neurons in mediating the negative feedback action of estrogen points towards the existence of yet another ERα-expressing partner, responsible for shaping the pattern of the GnRH/LH pulse.

In vitro studies using immortalized GnRH neurons have suggested an involvement of NO in the GnRH pulse generator. GT1-7 cells, which express a functionally active NOS protein, are synchronized by NO in a cGMP-dependent manner, promoting pulsatile GnRH secretion (López et al., 1997). In line with this, *in vivo* studies using gonadectomized rats have proposed an involvement of NO release in LH pulsatility (Rettori et al., 1993). Curiously, within the ARH, where the GnRH pulse generator is thought to exist, nNOS neurons have a unique GABAergic phenotype (Chachlaki, Malone et al., 2017) and are surrounded by kisspeptin fibers (Hanchate et al., 2012),). ARH GABA neurons play an important role in the control of progesterone-mediated negative feedback (Moore et al., 2015) and are able to promote robust LH secretion following optogenetic activation (Silva et al., 2019). In fact, within the ARH around 50% of GABA neurons in female mice express nNOS, whereas this co-expression is lower in males, reaching around 20% (Marshall et al., 2017). Thus, could both nNOS and kisspeptin neurons of the ARH collaborate to mediate the negative feedback of gonadal steroids dictating the clock ticking of the pulse generation?

Contrary to what has been observed in the MePO, the systemic administration of Kp10 fails to promote nNOS phosphorylation in the MBH (Hanchate et al., 2012). In addition, no evidence for the expression of active GPR54 has been found in nNOS neurons of the ARH using a transgenic LacZ knock-in mouse model (Hanchate et al., 2012). However, it should be noted that even though the LacZ technology may not be sufficiently sensitive to reveal the expression of GPR54 in the ARH (Herbison et al., 2010), GPR54 mRNA has been successfully detected by both *in situ* hybridization and PCR analysis within the ARH of primates, sheep and mice (Lee et al., 1999; Shahab et al., 2005; Fu & van den Pol, 2010; Smith et al., 2011). Further studies, using more sensitive techniques should be used to verify whether nNOS neurons of the ARH express GPR54 and respond to kisspeptin signaling. Interestingly, unidentified neurons within the ARH that do not express GPR54 have been shown to be either excited or inhibited by kisspeptin, indicating that kisspeptin may act through other postsynaptic receptors to communicate with neighboring neuronal populations (Liu & Herbison, 2015). Thus, it seems plausible that nNOS and kisspeptin cells also build communication networks using “non-traditional” pathways without involving the GPR54/AKT/P-nNOS pathway.

In line to this hypothesis, a recent study indicates that the kisspeptin/NO interaction provides a mechanism for the modulation of the refractory period of GnRH neurons after kisspeptin exposure, thus shaping GnRH pulsatility. More specifically, NO appears to re-enable the GnRH response to kisspeptin by terminating the calcium response and deactivating TRPC channels to restore their availability (Constantin et al., 2021). As illustrated in Figure 3 one could envision kisspeptin and nNOS neurons creating a microcircuit of excitatory and inhibitory inputs that together orchestrate GnRH release. During the estrogen negative feedback phase in diestrus, activation of KNDy neurons results in kisspeptin release, stimulating GnRH secretion. Concomitantly, activation of nNOS cells by kisspeptin would result in the production and diffusion of NO in the vicinity of GnRH neurons. NO would provide the “OFF” signal necessary for GnRH neurons to return to baseline, enabling them to respond to the next “ON” signal provided by kisspeptin, shaping in this way the LH pulse. The nNOS population involved in LH pulse, i.e. the source of NO, or its site of action remain vastly unknown. NO could operate such a switch by acting either directly on GnRH neurons (Clasadonte et al., 2008) or on its trans-synaptic inputs (Pielecka- Fortuna et al., 2008; Zhang et al., 2009; Pielecka-Fortuna and Moenter, 2010), or both. It would also be interesting to explore the possible involvement of nNOS neurons in kisspeptin-GnRH communication, as well as the possibility that NO also acts upstream of kisspeptin neurons. NO and kisspeptin could thus be the Yin and Yang of GnRH release, integrating and coordinating different neuronal afferents as parts of the GnRH pulse and the GnRH surge generators (Figure 3).

4.3 Interaction of nNOS and kisspeptin neurons as part of the "GnRH surge" generator

nNOS neurons located in the region of the OVLT, by lying outside the blood-brain-barrier (BBB), have unlimited access to circulating gonadal steroids and other molecules from the periphery (Langlet et al., 2013). OVLT nNOS neurons adjacent to GnRH neurons express ERα and are considered important candidates to convey peripheral information during the positive feedback phase (Chachlaki, Malone et al., 2017). Neuroanatomical studies in rodents, sheep and nonhuman primates have established an interconnecting network of nNOS/kisspeptin and nNOS/GnRH neuronal interactions, with kisspeptin fibers of the OV/MePO (originating from AVPV kisspeptin neurons) being closely apposed not only to GnRH cell somata but also to the densely clustered nNOS neuronal population of the OV/MePO (Herbison et al., 1996; Chachlaki, Malone et al., 2017; Bedenbaugh, McCosh et al., 2018).

The prevailing notion that the AVPV kisspeptin population is the main component of the GnRH surge generator is somehow unretentive as evidence shows that the initiation and completion of reproductive maturation can occur in the absence of kisspeptin/GPR54 signaling (Mayer & Boehm, 2011). In agreement to this, the selective restoration of GPR54 expression specifically in GnRH neurons in GPR54-null mice is not sufficient to restore the LH response upon administration of kisspeptin or other known activators of the gonadal axis (León et al., 2016), further confirming the involvement of non-GnRH kisspeptin-responsive cells of the POA in the modulation of the GnRH surge. In addition, central NMDA administration can elicit LH release in *Gpr54*- and *Kiss1*-null mice, acting, at least in part, through nNOS neurons (d'Anglemont de Tassigny et al., 2010). In support of the role of nNOS neurons in the control of the GnRH surge, the genetic ablation of nNOS (i.e. nNOS KO mice) or pharmacological inhibition of NOS activity specifically in the POA (i.e. by selectively knocking down PSD-95 and preoptic neuronal NO production) has been shown to disrupt ovarian cyclicity, blunting the LH surge (d'Anglemont de Tassigny et al., 2007). Furthermore, previous studies had already proposed that NO release could constitute a final modulatory event in the generation of the preovulatory GnRH surge (Bonavera et al., 1993, 1994).

Kisspeptin-induced NO release is potentially an essential part of the mechanism underlying the kisspeptin-dependent activation of GnRH neurons during the preovulatory surge. Interestingly, the frequency of NO pulses (i.e. ~10-20 Hz) is similar to that capable of optogenetically used to promote GnRH neuronal activity and/or secretion by kisspeptin neurons (Han et al., 2015; Qiu et al., 2016), suggesting that NO, kisspeptin and GnRH could act together in the form of a tripartite "KiNG" network to mediate LH secretion (Figure 4). During the estrogen positive feedback phase in proestrus, AVPV kisspeptin neurons, which

606 are strongly stimulated by estrogen, would in turn stimulate OV/MePO nNOS cells, resulting
607 in NO volume transmission in the vicinity of GnRH neurons. Diffusive homeostasis would
608 restrain GnRH activity en masse, allowing the sparse population of GnRH neurons to better
609 synchronize, priming them for their subsequent response to the estrogen-induced excitatory
610 kisspeptin stimulus, promoting thus LH surge.

611 To date, these evidences indicate that NO acts as a brake on GnRH production when
612 gonadal steroid negative feedback is operational. However, during the positive feedback
613 phase, this brake is first applied to and then released in a large number of GnRH neurons
614 simultaneously, as the same positive feedback action also continues to stimulate massive
615 kisspeptin production in the POA, leading to the surge of GnRH and LH. It seems plausible
616 from the above mechanism that the estrogen-induced kisspeptin-mediated activation of
617 nNOS neurons in proestrus could itself serve as a switch for the GnRH system, enabling the
618 transition between pulsatile and surge modes (Christian & Moenter, 2010).

619 **4.4 Insights into the involvement of the KiNG interactions controlling reproduction in**
620 **sheep and primates**

621 The GnRH system is highly conserved across mammalian species and the
622 neuroendocrine mechanisms controlling the ovine and primate ovarian cycle are similar in
623 many respects to those in rodents, in which the vast majority of studies have been carried
624 out over the last few decades. LH pulsatility, controlled by the negative feedback actions of
625 gonadal steroid hormones, occurs at a low level throughout the cycle in females and is
626 important for ovarian steroidogenesis. The LH surge, triggered by high circulating
627 concentrations of estradiol late in the follicular phase, promotes ovulation (Goodman &
628 Inskeep, 2015). However, differences in hypothalamic anatomy between species as well as
629 physiological imperatives such as seasonality impose some differences in the location,
630 identity and architecture of the neurons involved in LH pulsatility and LH surge as well as the
631 mechanisms of estrogen feedback. While the role of the “KiNG” network in shaping episodic
632 and non-episodic LH release has not been well studied, the data from other species does
633 support a strong relationship between the cell types involved.

634 In higher mammals, e.g. sheep, monkeys and humans, kisspeptin nerve terminals
635 reach the external zone of the ME, where the GnRH nerve terminals are found)
636 (Ramaswamy et al., 2008; Ramaswamy et al., 2010), while interestingly,
637 immunohistochemical observations of human hypothalamic tissue samples show that Dyn
638 expression is absent in kisspeptin neurons of the infundibular nucleus, the human equivalent
639 of the ARH (Hrabovszky et al., 2012). In both ewes and primates, as in rodents, the neural
640 elements necessary for episodic LH secretion are believed to reside in the MBH, and mostly

involve KNDy neurons present in the ARH (Goodman et al., 2013). Interestingly, in ewes, KNDy neurons do not express the GPR54 receptor (Smith et al., 2011), although GnRH neurons do (Smith et al., 2009). The GnRH pulse is thought to arise from an initial NKB-induced increase in the activity of KNDy neurons (through their expression of the NKB receptor, NK3R) (Amstalden et al., 2010), leading to the release of kisspeptin and the stimulation of GPR54-expressing GnRH neurons. Yet, the fact that the local administration of a GPR54 antagonist to the ARH of ovariectomized ewes decreases LH pulse frequency suggests that other kisspeptin-responsive cells (i.e. non KNDy neurons) found in the region could also be involved in shaping the LH pulse (Smith et al., 2011). A more recent study suggests that the feedback action of estrogen allowing for the onset of puberty and LH pulsatility in ewes involves ERα-expressing neurons, other than the ARH kisspeptin population (Bedenbaugh et al., 2018).

The possible involvement of the KiNG network in shaping LH pulsatility would be an interesting target of studies in sheep and primates, since neuroanatomical data from these species reveal that, similarly to rodents (Hanchate et al.,2012), ARH nNOS neurons are apposed closely by kisspeptin fibers (Bedenbaugh, McCosh et al., 2018). However, in contrast to rodents (Hanchate et al., 2012) and primates, in which kisspeptin and nNOS neurons belong to distinct neuronal populations, in ewes, almost all kisspeptin neurons in the ARH coexpress nNOS, regardless of age (prepubertal vs. postpubertal) or the luteal phase (Bedenbaugh et al., 2018; Bedenbaugh, McCosh et al., 2018; Bedenbaugh, O'Connell et al., 2018).

The hypothalamic mechanisms responsible for the activation of GnRH neurons over the preovulatory surge show important interspecific differences. These differences concern not only the hypothalamic sites involved in the LH surge, but also the mechanisms of estrogen positive feedback. In sheep, neurons located in the ventromedial hypothalamus (VMH) are responsible for mediating the estrogen positive feedback action, resulting in the LH surge (Caraty et al., 1998). Interestingly, a subset of somatostatin (SST)-nNOS neurons in the ventrolateral region of the VMH (vlVMH) that possibly coexpress ERα (Scanlan et al., 2003) is seen to be activated during the positive feedback phase of estrogen, and NO release from these neurons is suggested to be necessary for the preovulatory LH surge (McCosh et al., 2020). These subpopulations, in turn, activate GnRH neurons located in the POA and MBH, resulting in a prolonged GnRH surge. Close contacts between nNOS fibers and GnRH neurons have been evidenced across species, and the coexpression of nNOS in GnRH neurons has been seen in the POA of ewes as well as the MBH of rhesus monkeys (Bedenbaugh, McCosh et al., 2018), although such contacts have never been observed in rodents. Regardless of these differences however, it is evident that KNDy neurons and kisspeptin are not uniquely responsible for the generation of GnRH pulses and surge. Further

work is needed before the formulation of a "unifying hypothesis" that explains the mechanisms underlying these phenomena fundamental to the survival of species, while still taking into account their differences.

5 Concluding remarks and perspectives

Overall, the kisspeptin/nNOS neuronal network is ideally poised to generate and regulate episodic GnRH release in response to developmental and physiological cues, thus ensuring the precise sequence of events that constitutes pubertal activation and adult fertility (Choe et al., 2013; Messina et al., 2016). However, upstream of its release, GnRH must also be produced. Recent findings shed some light on the transcriptional mechanisms controlling the postnatal maturation of the GnRH system, apparently involving the action of microRNAs (miRNAs). miRNAs are short (~20-nucleotides) noncoding RNAs that silence gene expression in a post-transcriptional manner, principally by binding to the 3' untranslated regions (3'UTR) of target mRNAs, and have been previously implicated in the regulation of fertility at the level of peripheral organs (Papaioannou et al., 2010; Hasuwa et al., 2013). A multilayered miRNA-driven switch between inductive and repressive signals has been shown to govern GnRH expression during the transition from the infantile to the juvenile period, ensuring correct puberty onset (Messina et al., 2016; Heras et al., 2019). During minipuberty, this miRNA-driven epigenetic regulation of hypothalamic GnRH expression permits the sustained increase of neurohormone synthesis, a process believed to involve the tripartite KiNG network (Messina et al., 2016). In particular, miR-200/429, through its target Zeb1, affects the expression of GPR54, modulating the kisspeptin-mediated activation of GnRH expression, while in parallel, miR-155 counteracts NO/Cebpb-dependent GnRH repression (Messina et al., 2016). More recently, another hypothalamic miRNA-controlled pathway (miR-30) has been shown to influence the timing of puberty onset through the repression of a repressor of *Kiss1* transcription, Makorin ring finger protein 3 (Mkrn3) (Heras et al., 2019), suggesting that it could be worth exploring the upstream pathways regulating the components of the KiNG network.

Since kisspeptin is able to promote the activatory phosphorylation of the nNOS protein in the POA (Hanchate et al., 2012), one cannot exclude the possibility that kisspeptin is responsible for the increase in nNOS activity observed at minipuberty (Messina et al., 2016), or its involvement in the mechanisms promoting nNOS expression in the ARH during the infantile period (Chachlaki, Malone et al., 2017). Later on, the kisspeptin/nNOS network likely achieves some kind of homeostasis in order to establish the functionality of GnRH pulses and surges. A subset of afferents of KNDy neurons that display oscillatory activity have also been observed projecting to the POA (Bouret et al., 2004; Caron et al., 2012),

where these fibers are apposed to both GnRH and nNOS neurons in the OV/MePO, two populations that express GPR54 (Hanchate et al., 2012), suggesting more complex interactions between the different neuronal populations controlling GnRH pulsatile and surge release.

Overall, it appears at present that even though kisspeptin neurons undoubtedly play key role in GnRH secretion, they are not the sole arbiters or “monarchs” of the activation and function of the HPG axis, and that NO, by acting as a brake on GnRH neurons, not only leads to their pulsatility, but actually primes them for further stimulation, allowing kisspeptin to instigate the surge in neuropeptide release. Further studies are required to determine how NO and kisspeptin, by acting as the Yin and Yang of the GnRH axis, maintain reproductive homeostasis and the survival and propagation of the species.

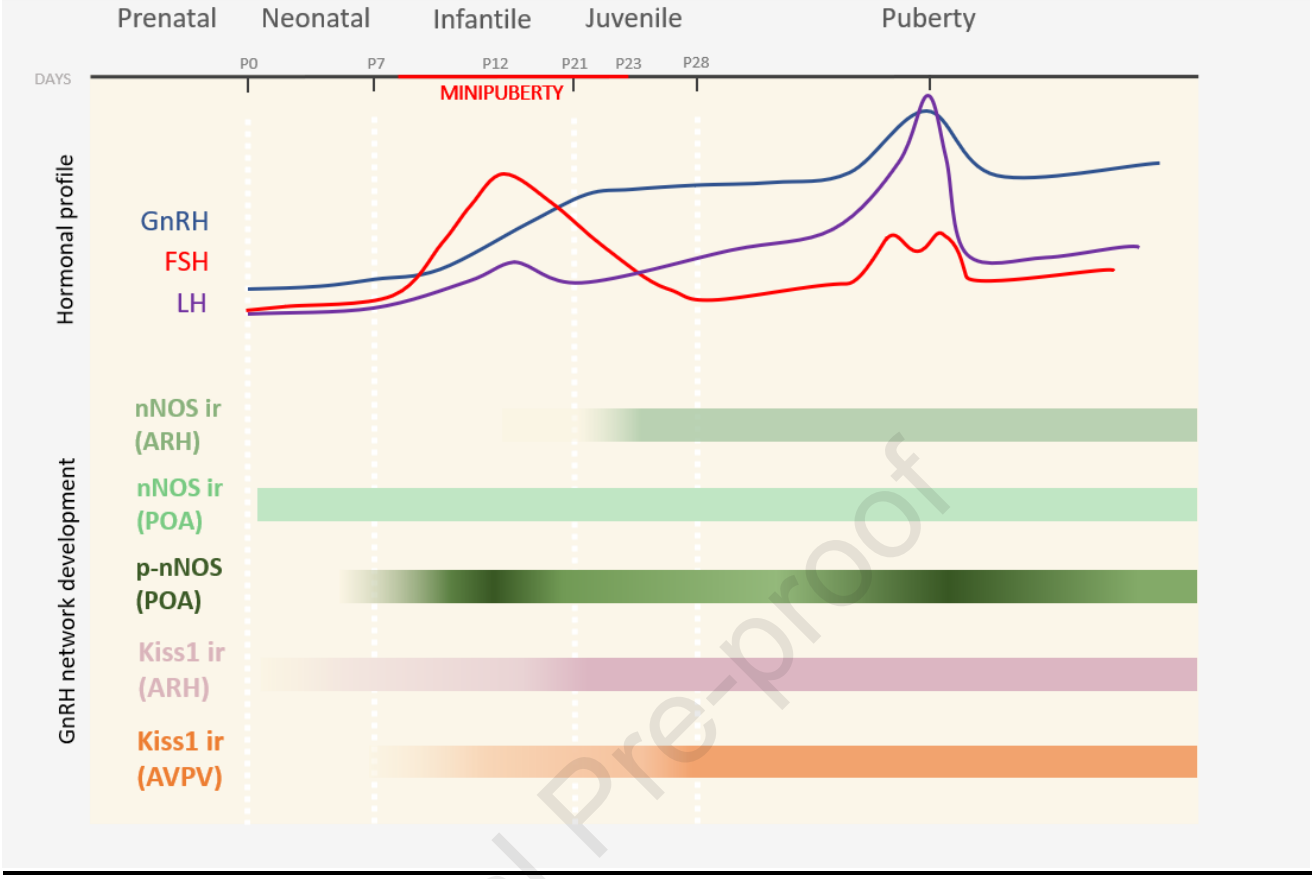

Figure 1. Development of GnRH neuronal network.

Postnatal development in rodents can be divided in four stages, all marked by developmental events: the neonatal (P0-P7), infantile (P8-P21), juvenile (P22-P30) and peripubertal period, ending with the initiation of puberty. GnRH peptide is already secreted at birth and it increases sharply during the second week of life marking the first postnatal activation of the hypothalamic-pituitary-gonadal (HPG) axis, known as minipuberty. The high levels of circulating estrogens, along with the lack of negative feedback on the neonatal GnRH pulse generator allows for the increase in the FSH levels until the completion of the infantile stage. With the initiation of puberty, the positive feedback action of gonadal steroids eventually results in the GnRH/LH surge. nNOS and kisspeptin neurons also follow distinct maturational patterns. nNOS immunoreactivity (ir) in the preoptic area (POA), within the organum vasculosum laminae terminalis (OV) and the median preoptic nucleus (MePO), is already observed at birth, remaining constant during development. Contrary to the nNOS-ir, the catalytic activity of the nNOS enzyme, marked by the levels of the nNOS phosphorylation on the positive regulatory site serine-1412, significantly increases concomitantly with minipuberty. During adulthood, nNOS phosphorylation significantly increases in proestrus, concomitant with the preovulatory increase in estrogen levels. In the arcuate hypothalamic nucleus (ARH) nNOS-ir appears only after the peak of minipuberty, being visible by the end

744 of the infantile stage. Kisspeptin-ir is already present at birth in the ARH, reaching adult
745 levels before the end of the infantile period. In the POA, within the region of the anteroventral
746 periventricular nucleus (AVPV), the first evidence of kisspeptin-ir is found around
747 minipuberty, with a massive increase in the following days, followed by a steady increase
748 until the end of the juvenile period. Adapted from Messina et al., 2016.
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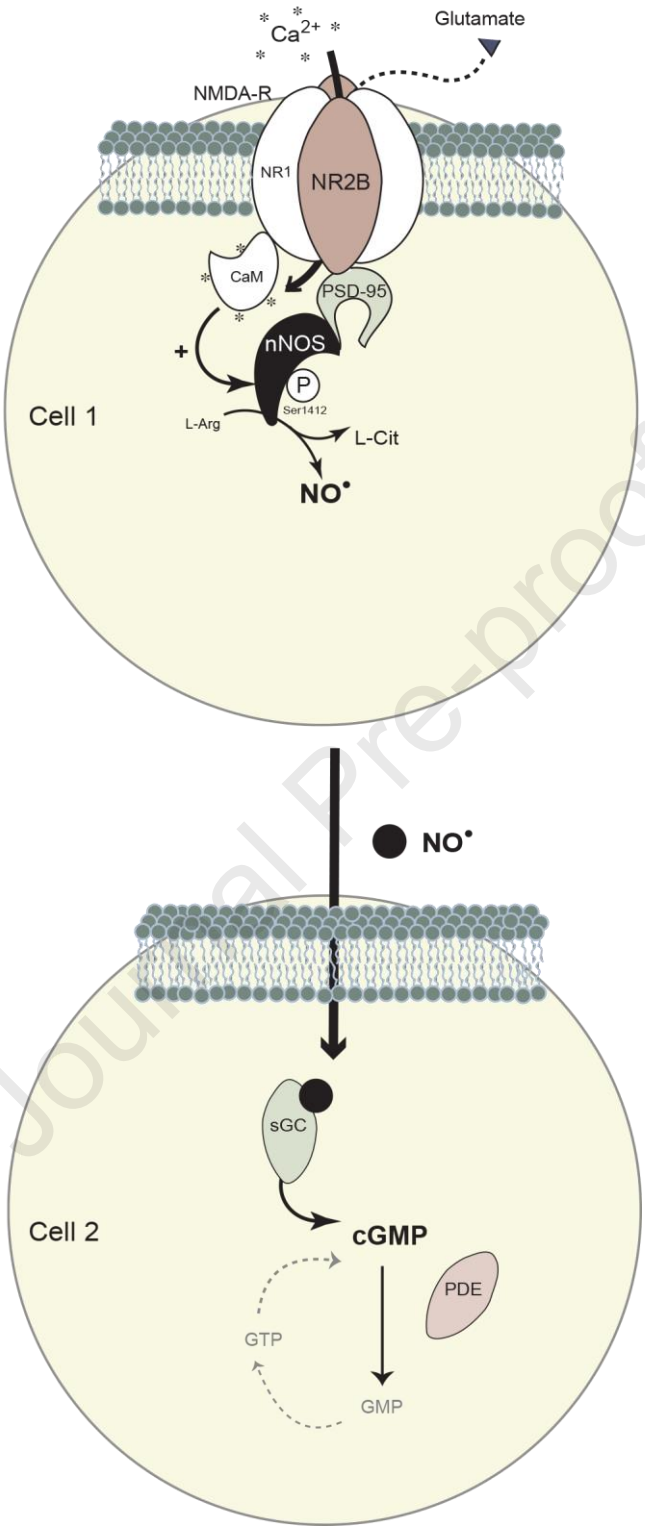
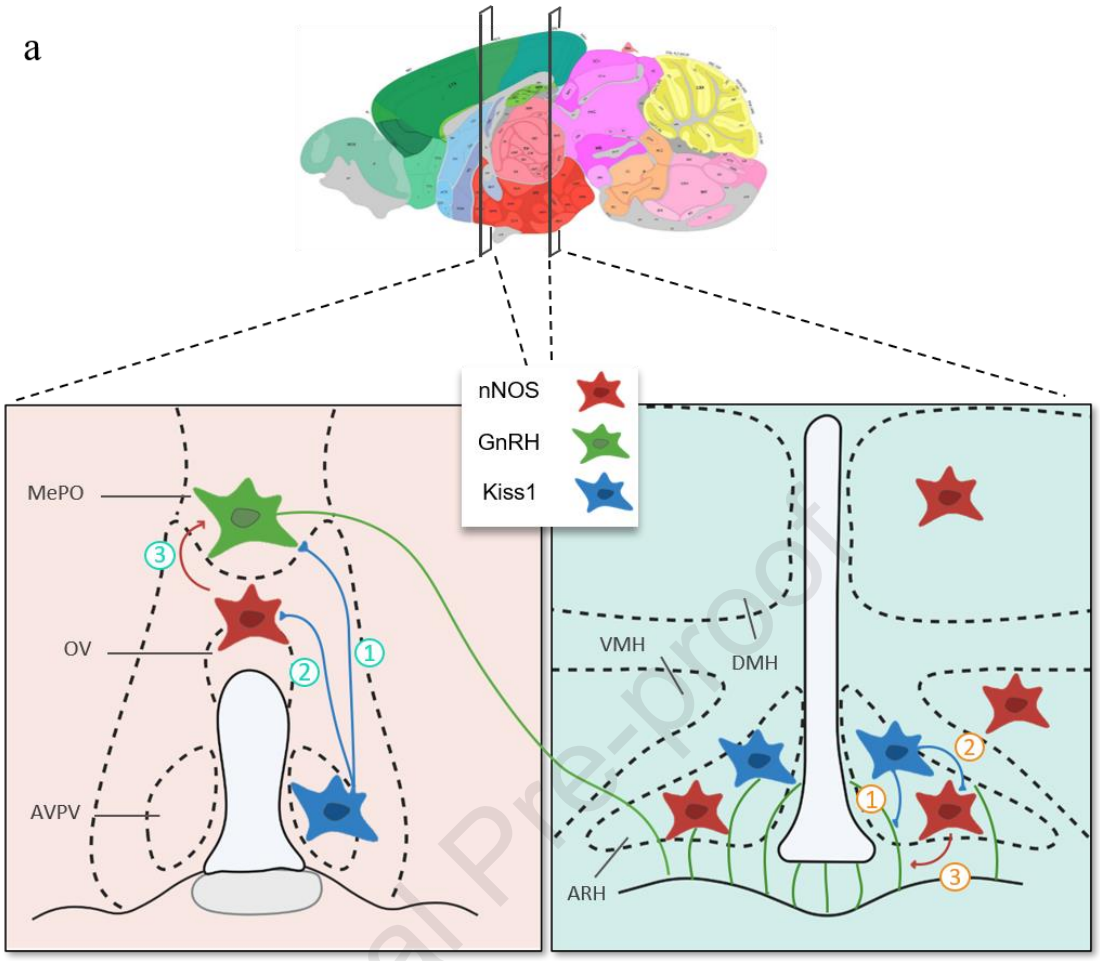


Figure 2. The NO production and downstream signaling cascade.

(Upper panel) The nNOS-PSD-95- NMDAR ternary complex. nNOS activation and the subsequent production of NO are dependent on the assembly of a ternary complex involving nNOS, the scaffolding protein post-synaptic density-95 (PSD-95) and the N-methyl-D-

aspartate (NMDA) receptor (NMDAR). The binding of glutamate to the NMDAR enables Ca^{2+} entry into the neuron which activates the nNOS (physically interacting with the NR2B subunit of the NMDAR) via the creation of a Ca^{2+} /calmodulin (CaM) complex. The activaton of nNOS results in the production of NO by the enzymatic conversion of L-arginine (L-Arg) to L-citrulline (L-Cit). In parallel, membrane-tethered nNOS is also subjected to post-transcriptional modifications, such as phosphorylation via protein kinase AKT at serine-1412, rapidly enhancing nNOS activity.

(Bottom panel) The NO–cGMP signalling pathway. Nitric oxide (NO) is a highly soluble and membrane-permeable neurotransmitter. Once NO is released it stimulates the production of the second messenger cyclic guanosine monophosphate (cGMP) by binding to soluble guanylate cyclase (sGC), inducing a conformational change that results in activation of the enzyme and the subsequent conversion of GTP to cGMP. cGMP the interacts with several intracellular targets like cGMP-binding phosphodiesterase (PDE), responsible for catalyzing the hydrolytic destruction of the cGMP to produce 5'-GMP. Considering that the biological and physiological effects of NO are influenced by its ambient concentration, i.e. the balance between its rate of synthesis and its rate of inactivation, the activity of the downstream effectors sGC and PDE is crucial for the cellular function in response to nNOS activation**.**



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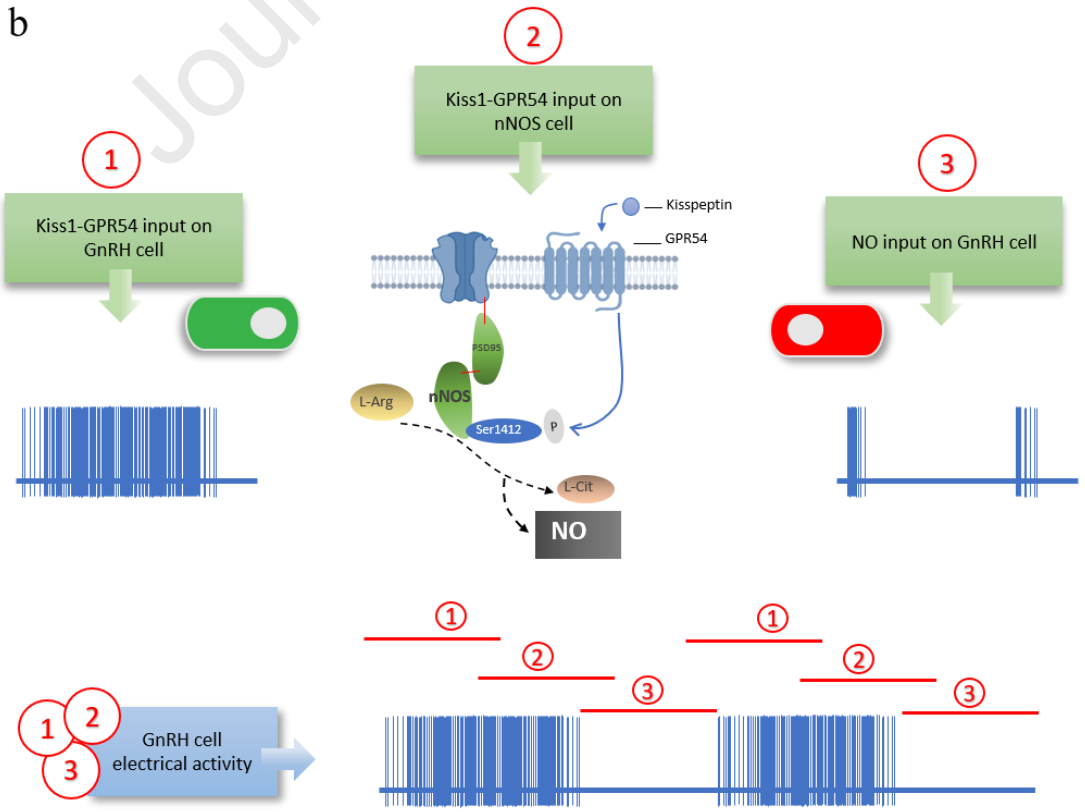

Figure 3. Involvement of the KiNG network in shaping GnRH pulsatile and surge release.

a) The KiNG network as part of the "GnRH pulse" and "GnRH surge" generators. Within the preoptic area (*left panel*), kisspeptin neurons (blue) and nNOS neurons communicate with the scattered GnRH neuronal cell bodies (green) located in the organum vasculosum laminae terminalis (OV) and the median preoptic nucleus (MePO). (1) Kisspeptin neurons of the anteroventral periventricular nucleus (AVPV) can both directly excite GnRH neurons (2) and promote the phosphorylation of nNOS in the OV/MePO, inducing its activation and subsequently NO production. Thereupon, NO may diffuse through volume transmission in the vicinity of GnRH neuronal cell bodies (3) acting as a brake to GnRH neurons, which may uphold their synchronous activity at the time of the preovulatory GnRH surge. Within the arcuate nucleus (ARH) (*right panel*) kisspeptin and nNOS neurons are in close proximity with the GnRH distal dendrites and terminals (green). (1) ARH kisspeptin neurons stimulate GnRH secretion; (2) concomitantly kisspeptin release may promote the activation of the nNOS population in the ARH. Production of NO in the vicinity of GnRH terminals (3) may inhibit GnRH neurons, contributing to the termination of GnRH/LH pulse. Considering ARH kisspeptin neurons can interact with the AVPV kisspeptin population we could also imagine that ARH kisspeptin neurons might indirectly interact with OV/MePO nNOS cells via their AVPV counterparts, hence promoting the action of NO at the level of GnRH neuronal soma.

(b) Proposed mode of action of the nNOS/ kisspeptin microcircuit during GnRH pulse. During the gonadal steroid hormone-mediated negative feedback, (1) ARH kisspeptin neurons activate the GPR54-expressing GnRH neurons promoting GnRH release. Kisspeptin also (2) acts on the GPR54-expressing nNOS neurons, promoting the activation of the nNOS enzyme triggering NO production. In turn, (3) NO acts on the GnRH neurons as the "OFF" signal necessary for GnRH neurons to return to their baseline activity; thus, enabling them to respond to forthcoming stimulus. This dynamic crosstalk driving depolarizing and hyperpolarizing responses in GnRH neurons (1, 2, 3) may give the pulse-like shape of the GnRH/LH release. Therefore, NO may operate as an important messenger for the estrogen-mediated switch from negative to positive feedback according to the sum of responses from the interaction between NO and kisspeptinergic signaling.

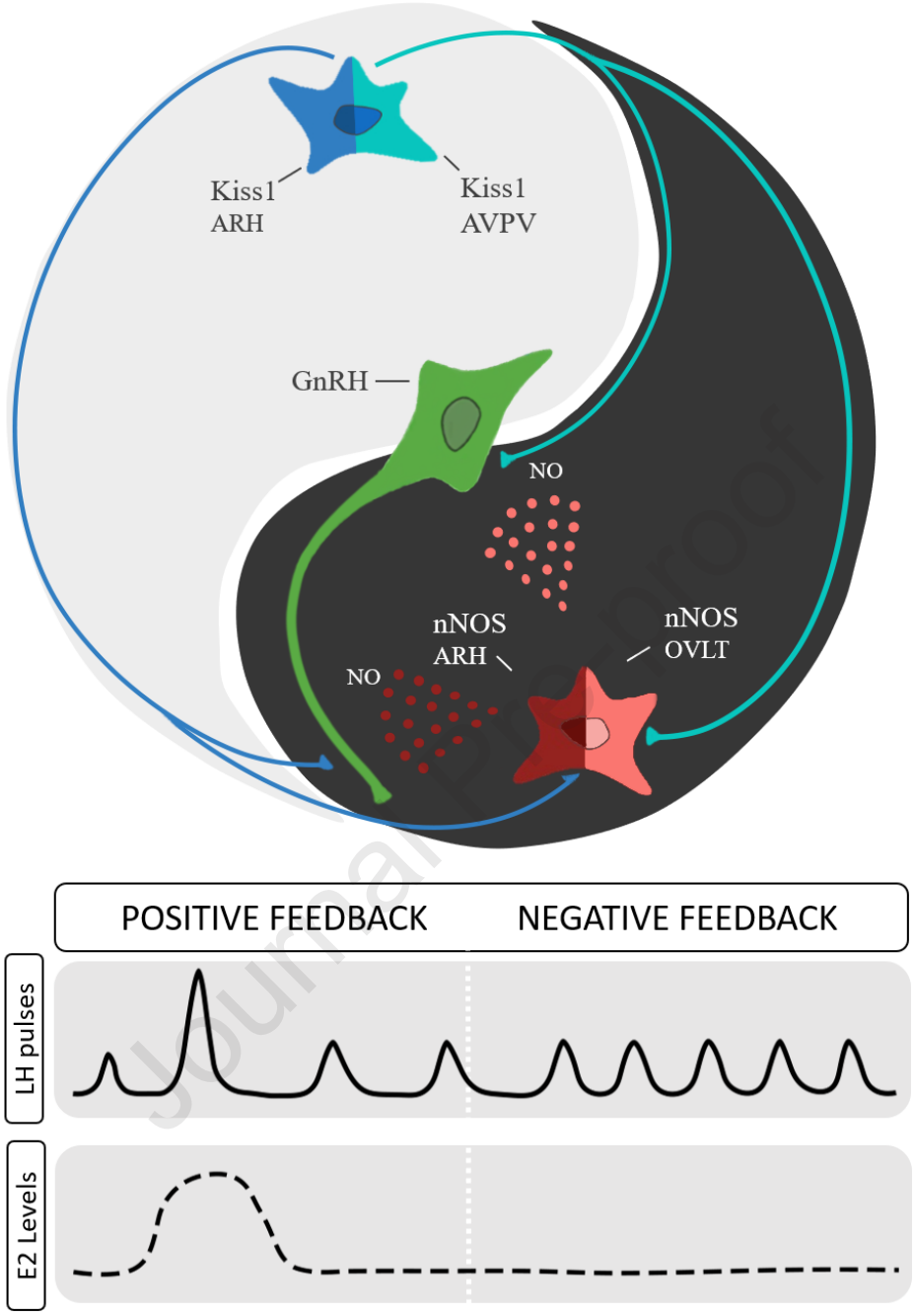

Figure 4. nNOS and kisspeptin as the Yin-Yang of the GnRH network.

In a concept of dualism, kisspeptin and nNOS neurons integrate and coordinate distinct signals in order to regulate GnRH secretion, driving the "GnRH pulse" and "GnRH surge" generators. The actions of the kisspeptin and nNOS populations seem to be counterbalanced, hence nNOS and kisspeptin could be represented as the Yin-Yang of GnRH network. NO may be synthetized by nNOS neurons in response to a kisspeptinergic drive in these neurons. During the positive feedback, high levels of estrogens stimulate the

AVPV kisspeptin population (light blue), promoting the estrogen-induced kisspeptin-mediated phosphorylation of the nNOS enzyme, inducing NO production by the nNOS cells located in the OV/MePO (light red). NO, transduced via volume transmission to the scattered GnRH population (green), restrains the activity of GnRH neurons, enabling their synchronization. With the activity of GnRH neurons being synchronous, the population of GnRH neurons becomes primed for their subsequent activation by kisspeptin, promoting the GnRH surge. During the negative feedback, ARH kisspeptin neurons (dark blue) stimulate GnRH release. Concomitantly kisspeptin also activates nNOS cells, resulting in the production and diffusion of NO that will provide the “OFF” signal for the GnRH neurons simultaneously, enabling them to return to baseline and thus restore their ability to respond to the next stimulatory kisspeptin signal. ARH kisspeptin neurons (dark blue) could interact (1) directly with ARH nNOS neurons (dark red), in turn acting upon GnRH nerve terminals, or (2) indirectly with POA nNOS neurons (light red) via the AVPV kisspeptin population (light blue), that would in turn act upon neighboring GnRH neuronal soma.

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The KiNG of reproduction: kisspeptin/ nNOS interactions shaping hypothalamic GnRH release

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- nNOS neurons are endowed with a modality switch, permitting local signaling when their activity is sparse or uncoordinated, and a volume signal when there is synchronous population activity
- Kisspeptin can promote nNOS phosphorylation, enabling nNOS synchronous activity and subsequently NO release
- NO has opposite effects to kisspeptin on GnRH secretion
- NO acts as a brake on GnRH production allowing GnRH neurons to better synchronize, priming them for the excitatory kisspeptin stimulus
- NO and kisspeptin, by acting as the Yin and Yang of the GnRH axis may shape GnRH pulses and surges