Expanding the role of tachykinins in the neuroendocrine control of reproduction

Chrysanthi Fergani and Víctor M Navarro

Department of Medicine, Division of Endocrinology, Diabetes, and Hypertension, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts, USA

Correspondence should be addressed to V M Navarro; Email: vnavarro@bwh.harvard.edu

Abstract

Reproductive function is driven by the hormonal interplay between the gonads and brain–pituitary axis. Gonadotropin-releasing hormone (GnRH) is released in a pulsatile manner, which is critical for the attainment and maintenance of fertility; however, GnRH neurons lack the ability to directly respond to most regulatory factors, and a hierarchical upstream neuronal network governs its secretion. We and others proposed a model in which *Kiss1* neurons in the arcuate nucleus (ARC), called as KNDy neurons, release kisspeptin (a potent GnRH secretagogue) in a pulsatile manner to drive GnRH pulses under the coordinated autocrine action of its cotransmitters, the tachykinin neurokinin B (NKB, stimulatory) and dynorphin (inhibitory). Numerous genetic and pharmacological studies support this model; however, additional regulatory mechanisms (upstream of KNDy neurons) and alternative pathways of GnRH secretion (kisspeptin independent) exist, but remain ill defined. In this aspect, attention to other members of the tachykinin family, namely substance P (SP) and neurokinin A (NKA), has recently been rekindled. Even though there are still major gaps in our knowledge about the functional significance of these systems, substantial evidence, as discussed below, is placing tachykinin signaling as an important pathway for the awakening of the reproductive axis and the onset of puberty to physiological GnRH secretion and maintenance of fertility in adulthood.

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Introduction

Successful production of offspring is indispensable to perpetuate species. As such, reproduction is under the control of a complex regulatory network, which involves the hypothalamic–pituitary–gonadal (H–P–G) axis. Gonadotropin-releasing hormone (GnRH) neurons, located in the hypothalamus, are a major component of the H–P–G axis and the ultimate regulators of reproductive function, including sexual behavior (Moenter *et al.* 2003, Herbison *et al.* 2008, Herbison 2016). Importantly, GnRH release is pulsatile, and even though GnRH neurons may display autonomous activity (spontaneous bursts), these do not seem to correlate with GnRH/LH pulses *in vivo* (reviewed in Navarro 2012). Furthermore, GnRH neurons lack the ability to sense most factors that influence reproductive function, such as endogenous signals (e.g. sex steroid hormones (Radovick *et al.* 2012, Hrabovszky & Liposits 2013, Roa 2013)) as well as environmental cues (e.g. stressors (Dobson *et al.* 2003)). Thus, a large body of research is now focusing on the discovery of higher hierarchy circuits and their efficacy in stimulating GnRH secretion into the hypophyseal portal vessels, thereby enabling gonadotropin (luteinizing hormone (LH) and

follicle-stimulating hormone (FSH)) secretion from the anterior pituitary into the peripheral circulation. From then on, LH and FSH reach the gonads to stimulate gametogenesis and sex steroid production. In turn, sex steroids exert positive and negative feedback effects on pituitary and hypothalamic target cells (Herbison 1998), completing the H–P–G axis. In this respect, over the past 10 years, several upstream neurophenotypes have been implicated in stimulatory and/or inhibitory regulation of GnRH secretion.

The path was initially paved with the discovery that loss-of-function mutations in several neuroendocrine genes, including *KISS1* and its receptor, *KISS1R* (Table 1), have been described to cause hypogonadotropic hypogonadism in humans (de Roux *et al.* 2003, Seminara *et al.* 2003, Chan *et al.* 2011, Topaloglu *et al.* 2012) due to a central deficit that leads to absent GnRH/LH pulses, highlighting the importance of these neural cues in GnRH release. Further anatomical and functional studies provided unequivocal evidence that kisspeptins, encoded by the *Kiss1* gene (Table 1), are the most potent secretagogues of GnRH in all mammals studied to date (Oakley *et al.* 2009). A number of studies by our lab and others suggest that *Kiss1* neurons, which contact GnRH neurons directly, receive profuse central and peripheral

Table 1 Protein and gene nomenclature for kisspeptin, tachykinins and their receptors, in human and rodent species.

Ligand	Gene encoding ligand		Highest affinity receptor	Gene encoding receptor	
	Human	Rodent		Human	Rodent
Kisspeptin	<i>KISS1</i>	<i>Kiss1</i>	Kisspeptin 1 receptor (GPR54)	<i>KISS1R</i>	<i>Kiss1r</i>
Substance P	<i>TAC1</i>	<i>Tac1</i>	Neurokinin receptor 1 (NK1R)	<i>TACR1</i>	<i>Tacr1</i>
Neurokinin A	<i>TAC1</i>	<i>Tac1</i>	Neurokinin receptor 2 (NK2R)	<i>TACR2</i>	<i>Tacr2</i>
Neurokinin B	<i>TAC3</i>	<i>Tac2</i>	Neurokinin receptor 3 (NK3R)	<i>TACR3</i>	<i>Tacr3</i>

regulatory inputs that modulate kisspeptin secretion for the initiation of puberty and the maintenance of fertility in adulthood (Seminara *et al.* 2003, Pinilla *et al.* 2012). Importantly, Kiss1 neurons also play a critical role in conveying information about the sex steroid milieu to GnRH neurons (Navarro *et al.* 2004, Gill *et al.* 2010). However, kisspeptin action on GnRH neurons is necessary but not sufficient for the proper activation of GnRH neurons (Leon *et al.* 2016).

The development of newer, more potent and less expensive tools to screen genome sequences of affected patients is revealing a growing number of factors that appear critical for the timing of puberty onset and maintenance of fertility by regulating kisspeptin and/or GnRH/LH release. Within this constellation of neuroendocrine systems is the one that comprises tachykinin neurokinin B (NKB) and its receptor (NK3R) encoded by *TAC3* and *TACR3* in humans respectively (Table 1). This system has received substantial attention as the identification in 2009 of inactivating mutations in these genes are also associated with hypogonadotropic hypogonadism and lack of puberty onset (Topaloglu *et al.* 2009, 2012, Young *et al.* 2010, Yang *et al.* 2012), resembling the phenotype of *KISS1/KISS1R*-null patients. Moreover, the systemic administration of an NK3R antagonist (ESN364) in OVX ewes, castrated or cycling nonhuman primates as well as healthy men and women (Fraser *et al.* 2015, 2016) show a partial inhibition of the reproductive axis. Indeed, numerous follow-up animal studies confirmed that NKB is a critical stimulatory input to the GnRH network in various species (Navarro 2013, Goodman *et al.* 2014) although, interestingly, this stimulatory effect is not observed in healthy men (Narayanaswamy *et al.* 2016), probably due to their circulating sex steroid levels as discussed below. However, unlike kisspeptin deficiency, the phenotype of patients lacking NKB signaling is less severe as reversal cases have been documented, in which some patients recovered reproductive function and fertility after delayed puberty (Gianetti *et al.* 2010). A similar subfertile phenotype has been observed in genetically modified mouse models, where *Tac2* and *Tacr3* (encoding NKB and NK3R respectively, in rodents, Table 1), had been deleted from the genome (Steiner & Navarro 2012, Yang *et al.* 2012, True *et al.* 2015). Therefore, it appears that the reversal phenotype in reproductive viability observed in human individuals with *TAC3/TACR3* or rodents with *Tac2/Tacr3* mutations may be due to compensation by other neuronal systems.

Interestingly, NKB is a member of the broader tachykinin family, which has the common C-terminal sequence of Phe-X-Gly-Leu-Met-NH₂ (Maggio 1988). This family also includes substance P (SP), neurokinin A (NKA), neuropeptide K (NPK) and neuropeptide γ (NP γ) (Otsuka & Yoshioka 1993, Page 2005). The vast majority of research has focused on SP, NKA and NKB, which bind preferentially to the NK1R, NK2R and NK3R G-protein-coupled receptors respectively (Maggi 1995, Patacchini & Maggi 2001, Saffroy *et al.* 2003).

Early studies documented a robust stimulatory action of LH release by SP in rats, rabbits and humans (Arisawa *et al.* 1990, Coiro *et al.* 1992, Kalra *et al.* 1992, Sahu & Kalra 1992, Traczyk *et al.* 1992), and recent electrophysiological studies have described potent depolarizing effects of SP and NKA on ARC Kiss1 neurons in the mouse (de Croft *et al.* 2013) indicating that LH stimulation by these tachykinins involves, at least in part, a kisspeptin-dependent mechanism. Of note, this study showed that, *in vitro*, the activation of kisspeptin neurons by NKB was completely diminished only when all three neurokinin receptor (NKR) subtype-selective antagonists were concomitantly applied in the *in vitro* bath (de Croft *et al.* 2013). This is in line with studies carried out *in vivo* indicating that blockade of all 3 tachykinin receptors (but not each one of them individually) prevented the compensatory rise of LH after gonadectomy (GDX) in rats (Noritake *et al.* 2011). Therefore, considerable cross-reactivity exists between these receptor/ligand systems, and each one of these neuropeptides is capable of eliciting responses from all three neurokinin receptors (Cascieri *et al.* 1992, Gether *et al.* 1993, Beaujouan *et al.* 2000). In these studies, the affinities or EC₅₀ values of each tachykinin for NK1R, NK2R and NK3R respectively were reported as follows: SP₂ nM, 2200 nM and 18,000 nM; NKA₁₆ nM, 3 nM and 1300 nM and NKB₇₀ nM, 25 nM and 4 nM (Seabrook *et al.* 1995). These data suggest a likely interaction of NKA with NK1R as well as NK2R, and of NKB with all 3 receptors, at relatively low concentrations. Furthermore, it has been demonstrated in rats that pulsatile LH secretion was suppressed by central administration of CS-003, an antagonist for all three NKRs, whereas administration of each NKR subtype-selective antagonist alone had no effect (Noritake *et al.* 2011). In this respect, several pieces of evidence will be discussed below that provide unequivocal evidence that other members of the tachykinin family, namely SP and NKA, all encoded by the *TAC1* or *Tac1* gene (Table 1),

in humans and rodents respectively (Lasaga & Debeljuk 2011) are an important component of the integrated neuronal hypothalamic system that controls GnRH/LH secretion in mammals.

The current model for the GnRH pulse generator

Kiss1 neurons are located primarily in two discrete hypothalamic nuclei: the arcuate nucleus (ARC) and the anteroventral periventricular nucleus (AVPV/PeN) in rodents (Clarkson *et al.* 2009) or the preoptic area in ruminants (Lehman *et al.* 2010), monkeys (Luque *et al.* 2011) and humans (Hrabovszky 2014). Compelling evidence suggests that Kiss1 neurons in the ARC mediate the negative feedback of sex steroids, and *Kiss1* expression is inhibited by estradiol (E_2) and testosterone (T). By contrast, *Kiss1* expression in the AVPV/PeN, almost exclusive to the female brain, is upregulated by E_2 and mediates the positive feedback that leads to the female-specific preovulatory GnRH/LH surge (Navarro *et al.* 2004, Smith *et al.* 2005, Maeda *et al.* 2007). Substantial *in vivo* and *in vitro* evidence points to the importance of a population of neurons located in the ARC of the hypothalamus in playing the role of the GnRH pulse generator. The notion originated from studies carried out in the ovariectomized (OVX) rhesus monkey, in which LH secretion was abolished by selective lesioning of the ARC (Plant *et al.* 1978) and was further reinforced by findings that multiunit electrical activity (MUA) in the vicinity of ARC kiss1 neurons was tightly coupled LH pulses (Kawakami *et al.* 1982, Ohkura *et al.* 2009). In this context, Kiss1 neurons in the ARC coexpress dynorphin (inhibitory) and NKB (stimulatory) referred to as KNDy neurons (Cheng *et al.* 2010, Navarro 2012, Goodman *et al.* 2013), which have been proposed to act in a coordinated, reciprocal fashion to shape the pulsatile release of kisspeptin in the median eminence, which in turn induces corresponding intermittent GnRH discharges at this site (Keen *et al.* 2008). This has since been demonstrated in a variety of mammals including mice (Navarro *et al.* 2009), rats (Navarro *et al.* 2011a), sheep (Goodman *et al.* 2013), goats (Wakabayashi *et al.* 2010) and monkeys (Ramaswamy *et al.* 2010). In this model, NKB would stimulate kisspeptin release and dynorphin would then inhibit this release through autocrine loops, thus shaping a kisspeptin/GnRH/LH pulse (Keen *et al.* 2008). This is supported by the anatomical findings that virtually all KNDy neurons express NK3R (Navarro *et al.* 2009, 2011b, Amstalden *et al.* 2010) and >90% express kappa-opioid receptor (KOR; (Weems *et al.* 2016)). Furthermore, KNDy cells are interconnected with NKB fibers within the ARC forming a tightly regulated network (Rance & Bruce 1994, Krajewski *et al.* 2010, Lehman *et al.* 2010). Indeed, a growing number of studies in multiple species from our lab and others support the ability of NKB, or the NKB receptor (NK3R) agonist senktide, to increase LH pulses

(Grachev *et al.* 2012, Navarro 2013, Goodman *et al.* 2014). This places the KNDy neurons as ideal candidates for the role of the GnRH pulse generator. However, more recently, several studies have provided evidence that other tachykinins, i.e., SP and NKA, merit further investigation as additional fundamental components of the current, KNDy-dominated, GnRH pulse generator model. Although no human mutations in the genes encoding SP and NKA (*TAC1*) or their receptors (*TACR1* and *TACR2* respectively; Table 1) have been correlated with reproductive disorders yet, both SP and NKA have been reported to stimulate the gonadotropic axis in several species (Arisawa *et al.* 1990, Kalra *et al.* 1992, Sahu & Kalra 1992, Noritake *et al.* 2011, de Croft *et al.* 2013, Navarro *et al.* 2015) including men (Coiro *et al.* 1992). It is therefore plausible to speculate that these tachykinins are involved in the central regulation of GnRH release and may be additional elements to the GnRH pulse generator.

Anatomical studies

The topographical identification of tachykinin ligands and their receptors has provided important insight in to the potential mechanisms of action of these systems for the control of GnRH/LH secretion. Several studies using *in situ* hybridization, immunohistochemistry and single-cell RT-PCR for the detection of mRNA and protein of tachykinins and their receptors, as well as their morphological relationship to Kiss1 and GnRH neurons, have been carried out to date. However, important information, especially regarding the localization of receptors, across a large number of species, is still lacking.

Distribution of SP and NKA in the hypothalamus and anatomical relationship with Kiss1 and GnRH neurons

Within the hypothalamus, the largest population of NKB immunoreactive cells has been detected in the ARC (and specifically in the middle to caudal aspects) with smaller numbers identified in the ME, POA, lateral septum, bed nucleus of the stria terminalis, amygdala and the paraventricular nucleus of rats, sheep and mice (Rance & Young 1991, Goubillon *et al.* 2000, Navarro *et al.* 2009). The ARC population has received most attention, as in this nucleus, kisspeptin and NKB reside in the same cell (KNDy; (Goodman *et al.* 2007, Navarro *et al.* 2009)), whereas no instances of NKB and GnRH colocalization have been reported, although GnRH and NKB immunopositive fibers have been observed to interweave in the rat ME (Krajewski *et al.* 2005).

In mice, *Tac1* mRNA (encoding SP and NKA) has been mapped out in the brain of female mice using *in situ* hybridization (Navarro *et al.* 2015). Within the hypothalamus, expression was found to be concentrated mainly in 2 regions: the ARC (especially the caudal aspect)

and the ventromedial nucleus (VMN), in keeping with previous reports of SP immunoreactivity in rats, monkeys and humans (Ronnekleiv *et al.* 1984, Yamano *et al.* 1986, Harlan *et al.* 1989, Rance & Young 1991, Tsuruo *et al.* 1991, Rance & Bruce 1994, Borsay *et al.* 2014). Studies using immunohistochemical detection of SP also report a plethora of fibers that innervate the entire length of the ARC and the median eminence (ME) (Hrabovszky *et al.* 2013, Kalil *et al.* 2015, Fergani *et al.* 2016), which appear to surround the capillaries of the hypophyseal portal system indicating that SP may have the ability to act directly on the anterior pituitary (Kalil *et al.* 2015).

Interestingly, even though the *Tac2* (gene encoding NKB; Table 1) is known to be coexpressed within Kiss1 in the ARC of various species, including humans (Goodman *et al.* 2007, Navarro *et al.* 2009, Hrabovszky 2014), the *Tac1*-positive neurons did not colocalize with *Kiss1*-positive neurons in the mouse (Navarro *et al.* 2015; Fig. 1). This is in agreement with equivalent investigations in the sheep Fergani *et al.* 2016), monkey (Kalil *et al.* 2015) and rat (Rance & Bruce 1994) but contradict findings in the human that report that approximately 65% of SP neurons in the ARC coexpress kisspeptin (conversely, 30% of Kiss1 neurons contain SP; (Hrabovszky *et al.* 2013)). The reason for this divergence is not known, however, it supports the notion for the existence of

potential differences in the function of the tachykinin systems across species (Hrabovszky *et al.* 2013, Kalil *et al.* 2015, Navarro *et al.* 2015). Nonetheless, the population of *Tac1* neurons in the ARC of the mouse (Navarro *et al.* 2015) and SP immunoreactive neurons and fibers in the monkey (Kalil *et al.* 2015) appeared to be in close contact with Kiss1 neurons and fibers (and GnRH fibers as shown in postmenopausal women (Hrabovszky *et al.* 2013)) in the ARC, presumably facilitating the interaction between all three neuronal populations. Immunohistochemical analysis of NKA fiber colocalization with kisspeptin or GnRH afferents merits future investigation. Of note, *Tac1* mRNA was not detected in the AVPV/PeN of mice (Fig. 1) or the POA of sheep Fergani *et al.* 2016), respectively, the regions in which the second population of Kiss1 neurons reside (Oakley *et al.* 2009); however, data from other species are non-existent.

Distribution of NK1R and NK2R in the hypothalamus and anatomical relationship with Kiss1 and GnRH neurons

Single-cell RT-PCR analysis of the expression of all 3 tachykinin receptors (*Tacr1*, *Tacr2* and *Tacr3* mRNA; Table 1) in Kiss1 (ARC and AVPV/PeN) and GnRH neurons showed that almost half (~49%) of Kiss1 neurons in the ARC and over one-fourth (~27%) of Kiss1 neurons in the AVPV/PeN express *Tacr1* mRNA, which is also present in a subset of GnRH neurons (~23%; (Navarro *et al.* 2015)). By contrast, in the sheep, NK1R protein was detected infrequently in kisspeptin neurons (approximately 6% of kisspeptin neurones coexpressed NK1R) in the ARC, but not in Kisspeptin or GnRH cells in the POA Fergani *et al.* 2016) *Tacr2*, was absent from both populations of Kiss1 neurons and GnRH neurons in mice (Navarro *et al.* 2015). Finally, *Tacr3* was confirmed to be present in all (100%) ARC Kiss1 neurons but minimally present (~10%) in AVPV/PeN Kiss1 neurons of mice, as has been previously described in various species (Navarro *et al.* 2009, 2015, Amstalden *et al.* 2010). Of note, *Tacr3* mRNA was also detected in a small subset of GnRH neurons (~11%; (Navarro *et al.* 2015)) as has been previously been reported in the rat (16% of GnRH somata contained NK3R immunostaining) (Krajewski *et al.* 2005) but not in the sheep (Amstalden *et al.* 2010, Ahn *et al.* 2015). In addition, extensive colocalization between GnRH axons with NK3R-positive fibers have been reported in the ME and organum vasculosum of the lamina terminalis of the rat (Krajewski *et al.* 2005), whereas >70% GnRH neurones are contacted by NK3R presynaptic terminals in the sheep Ahn *et al.* 2015). Whether NK1R or NK2R is expressed in KNDy and/or GnRH neurons in other species is unknown.

Taken together, these anatomical data allow us to postulate that SP can regulate GnRH secretion not only

Figure 1 Schematic representation of a hypothalamic neuronal network comprising Kiss1 neurons, GnRH neurons and *Tac1* neurons in the mouse. Percentage data depicting the co-expression of each receptor at each neuronal population as observed in studies carried out in mice using single-cell RT-PCR (Navarro *et al.* 2015). ARC Kiss1 neurons (KNDy neurons) are able to respond to NKB and half of them can also respond to SP. A subset of AVPV/PeN Kiss1 neurons also expresses the receptor for SP (NK1R) and a small fraction of them also express NKB receptor (NK3R). In addition, GnRH neurons, which respond primarily to kisspeptin, express SP and NKB receptors in small numbers. Finally, NKA must act on yet unknown intermediate neurons to stimulate kisspeptin release. Note: the location of the receptors in the cell (soma vs terminals) in this model, as well as the location of NKA-responsive neurons, is merely hypothetical.

indirectly by initial action on Kiss1 neurons but also directly by acting on GnRH neurons, although functional evidence for this pathway is lacking. Furthermore, the existence of axo-axonic or axo-dendritic synapses between SP and Kiss1 or GnRH axons remains to be elucidated. In the human, where SP and kisspeptin have been shown to colocalize, autocrine/paracrine actions of SP on KNDy neurons are also probable (Hrabovszky *et al.* 2013). Intriguingly, in the mouse, a subset of AVPV/PeN Kiss1 neurons are also receptive to SP actions (one-fourth of these cells contain NK1R), and it is well known that this population is involved in the generation of the GnRH/LH surge (Oakley *et al.* 2009). Therefore, a role for SP signaling in the shaping of the GnRH surge is likely, but remains unexplored. The action of NKA, on the other hand, remains largely unresolved because *Tacr2* has been identified in neither Kiss1 nor GnRH neurons, thus suggesting the presence of unidentified intermediate upstream neurons ((Navarro *et al.* 2015); Fig. 1).

Sex steroid regulation of SP and NKA

All known cotransmitters present in ARC Kiss1 neurons (Kiss1, NKB and dynorphin) are inhibited by sex steroids as part of their hypothesized role in the negative feedback upon GnRH release (Gottsch *et al.* 2009, Navarro *et al.* 2009). This also appears to be true for SP and NKA, as *Tacr1*-expressing neurons in the ARC and VMN of mice were downregulated by OVX and E₂ treatment (Micevych *et al.* 1988, Navarro *et al.* 2015) and immunopositive SP protein in the ARC increased after gonadectomy (GND) in the male monkey (Kalil *et al.* 2015). Furthermore, this effect appeared to be specific for these areas of the brain (Navarro *et al.* 2015) and was not evident elsewhere. Similarly, SP mRNA increased in the hypothalamus of post-menopausal compared to pre-menopausal women (Rance & Young 1991), and the content of SP in the ARC has been shown to increase after OVX in the rat (Tsuruo *et al.* 1987). The results of all these studies suggest that downregulation of SP and NKA in hypothalamic neurons may mediate, at least in part, the negative feedback action of gonadal steroids on gonadotropin secretion. Indeed, earlier studies have demonstrated that a substantial population of SP-immunoreactive cells located in the mediobasal hypothalamus of the rat are estrogen receptive (26.1% in the Arc and 42.9% in the VMN) (Akesson & Micevych 1988). Interestingly, immunohistochemical studies on human hypothalami have revealed that postmenopausal women have higher numbers of SP neurons and darker labeling than in age-matched men (Hrabovszky *et al.* 2013). However, if this constitutes a sex difference in the expression of SP or it is a mere reflection of different levels of sex steroids remains to be elucidated. In this context, an earlier report documents greater SP immunoreactivity in the medial amygdala of male compared to female rats (Micevych *et al.* 1988), an area which is also known for a greater

Kiss1 population of cells in males vs females (Stephens *et al.* 2016). However, the interaction between these two systems (SP and Kiss1 in the medial amygdala) has not yet been explored. Nonetheless, sex differences in the expression of SP or NKA require further characterization across multiple species.

Regulation of LH release by tachykinins: sex steroid-dependent action

Neurokinin B

Most studies carried out to date looking into the effect of tachykinins on reproductive function have focused on the role of NKB and less so on other members of the tachykinin family. Therefore, it is useful to compare findings from SP and NKA studies with those already carried out for NKB, as a synergistic action is highly probable. One thing that can be said about the stimulatory effect of NKB on LH release is that it is less robust than that of kisspeptin, and inhibitory actions or null effects on LH secretion have also been documented, depending on the species and the sex steroid levels (Sandoval-Guzman & Rance 2004, Navarro *et al.* 2011a, Ruiz-Pino *et al.* 2012). For instance, NKB induced significantly stimulatory LH responses in adult female rats and mice under physiological levels of sex steroids, whereas only adult intact male mice (but not rats) displayed LH responses to the same challenge (Navarro *et al.* 2011b, Ruiz-Pino *et al.* 2012). By contrast, predominant inhibitory effects of the selective NK3R agonist, senktide, have been reported in rodents with null or low sex steroids levels (Sandoval-Guzman & Rance 2004, Navarro *et al.* 2009, 2011b, 2015, Grachev *et al.* 2012), even though kisspeptins are known to stimulate gonadotropin secretion irrespective of the sex steroid milieu (Oakley *et al.* 2009). From a mechanistic point of view, the inhibitory action of NKB on LH release appears to be opioid mediated, as has been shown by the lack of LH inhibition by senktide in the presence of KOR agonist in rats (Kinsey-Jones *et al.* 2012). In accordance, extracellular recordings from KNDy neurons demonstrated that gonadal feedback (by both estrogen and dihydrotestosterone) attenuates the stimulatory effects of senktide on the firing rate of KNDy neurons while increasing the inhibitory effects of dynorphin by modulating the activation of NK3R and KOR (Ruka *et al.* 2016). Interestingly, in the sheep, NKB/NK3R signaling may also be important in the generation of the preovulatory GnRH/LH surge. For example, intracerebroventricular (i.c.v.) microinjections of senktide, in this species, results in a surge-like elevation of LH during the follicular but not the luteal phase of the ovine estrous cycle (Billings *et al.* 2010, Porter *et al.* 2014), replicating a potential dual effect of NKB, dependent on sex steroid levels, as observed in rodents (Navarro *et al.* 2011a).

These observations illustrate the complexity of the effects of NKB on the gonadotropic axis.

Substance P

To date, SP has largely been associated with processes unrelated to reproductive function, such as pain perception and inflammatory activity in the brain (De Felipe *et al.* 1998) as well as with psychiatric disorders (Ebner & Singewald 2006). Even though SP was originally identified in the 1930s (Lasaga & Debeljuk 2011), it is only now beginning to come in to the spotlight as a regulator of the reproductive axis. Few earlier studies aimed to investigate the effects of SP on the gonadotropic axis and report variable results (Table 2). These include peripheral (i.v.) administration of SP for 1 hour in normal men, which induced a robust discharge of LH (Coiro *et al.* 1992), and in OVX rats, i.c.v.-specific antiserum against SP (anti-SP) decreased plasma LH, whereas synthetic SP injected i.c.v. or i.v. into OVX+E₂ rats stimulated LH release by both routes of administration (Arisawa *et al.* 1990). Other studies conducted by Kalra and coworkers in the 90s (Kalra *et al.* 1992, Sahu & Kalra 1992) report null or inhibitory effects in intact and GND males respectively, hinting at potential sex differences in response to SP (Table 2). Further studies conducted on intact and OVX rabbits report that although the stimulatory effect of SP on LH is sex steroid independent, in the absence of ovarian steroids, SP is stimulatory only during the rising phase

of an LH pulse (Traczyk *et al.* 1992). Interest in SP has recently rekindled and studies in mice are pointing toward a clear stimulatory action on LH secretion, which appears to be independent of the sex steroid milieu (Table 2; (Navarro *et al.* 2015)). In this study, the activation of NK1R with the i.c.v. administration of an NK1R-specific agonist (GR73632) induced LH release in intact males, diestrous or OVX females and a 20-fold increase in OVX+E₂ females (Navarro *et al.* 2015). However, in rats that received the same agonist i.c.v., with the same dose, no alteration in LH levels was observed in either sex with intact gonads (Ruiz-Pino *et al.* 2015) indicating a potential species difference. This notion is also supported by pharmacological data from ovary-intact anestrous ewes and OVX and OVX+E₂ goats demonstrating that much higher doses of SP are needed to stimulate LH secretion compared to those needed with NKB or senktide (Yamamura *et al.* 2015, Fergani *et al.* 2016).

In addition, a small body of literature has focused on the role of SP on the LH surge as well as sexual behavior. Intriguingly, a number of reports by Kerdelhué and coworkers, in humans, monkeys and rats have shown variable results. Initially, a study carried out in cycling rats investigated the effects of a subcutaneous injection of SP during proestrus, which led to a reduction of the LH surge amplitude (Duval *et al.* 1996). Furthermore, this inhibitory effect was reversed with the simultaneous administration of SP and an NK1R antagonist (RP 67580) (Duval *et al.* 1996). However, further studies showed a

Table 2 Summary of results from *in vivo* studies investigating the effects of Substance P on LH secretion in various species.

Species	Sex	Age	Drug	Gonadal status	Route	LH	Reference
With the presence of sex steroids							
Human	Male	Adult	SP peptide	Intact	i.v. for 1 h	Stimulation	Coiro <i>et al.</i> (1992)
Rat	Female	Adult	SP peptide	OVX+E ₂	i.c.v.	Stimulation	Arisawa <i>et al.</i> (1990)
Rat	Female	Adult	SP peptide	OVX+E ₂	i.v.	Stimulation	Arisawa <i>et al.</i> (1990)
Rat	Male	Adult	SP peptide	Intact	i.c.v.	No effect	Kalra <i>et al.</i> (1992)
Rabbit	Female	Adult	SP peptide	Intact	i.c.v.	Stimulation	Traczyk <i>et al.</i> (1992)
Rabbit	Female	Adult	SP peptide	End of LH pulse	i.c.v.	No effect	Traczyk <i>et al.</i> (1992)
Mouse	Male	Adult	GR73632 (NK1R-Agonist)	Intact	i.c.v.	Stimulation	Navarro <i>et al.</i> (2015)
Mouse	Female	Adult	GR73632 (NK1R-Agonist)	OVX+E ₂	i.c.v.	Stimulation	Navarro <i>et al.</i> (2015)
Rat	Female	Adult	GR73632 (NK1R-Agonist)	Intact	i.c.v.	No effect	Ruiz-Pino <i>et al.</i> (2015)
Rat	Male	Adult	GR73632 (NK1R-Agonist)	Intact	i.c.v.	Stimulation	Ruiz-Pino <i>et al.</i> (2015)
Sheep	Female	Adult	SP peptide	Intact (anestrous)	i.c.v.	Stimulation	Goodman <i>et al.</i> (2015)
Goat	Female	Adult	GR73632 (NK1R-Agonist)	OVX+E ₂	i.v.	No effect	Yamamura <i>et al.</i> (2015)
Without the presence of sex steroids							
Rat	Female	Adult	Anti-SP peptide	OVX	i.c.v.	Inhibition	Arisawa <i>et al.</i> (1990)
Rat	Female	Adult	Anti-SP peptide	OVX	i.v.	No effect	Arisawa <i>et al.</i> (1990)
Rat	Male	Adult	SP peptide	Castrated	i.c.v.	Inhibition	Kalra <i>et al.</i> (1992)
Rat	Female	Adult	SP peptide	OVX	i.c.v.	No effect	Sahu and Kalra (1992)
Rat	Female	Adult	(Sar9, Met(O2)11)SP (NK1R-Agonist)	OVX	i.c.v.	No effect	Sahu and Kalra (1992)
Rabbit	Female	Adult	SP peptide	OVX	i.c.v.	Stimulation	Traczyk <i>et al.</i> (1992)
Mouse	Female	Adult	GR73632 (NK1R-Agonist)	OVX	i.c.v.	Stimulation	Navarro <i>et al.</i> (2015)
Goat	Female	Adult	GR73632 (NK1R-Agonist)	OVX	i.v.	Stimulation	Yamamura <i>et al.</i> (2015)
Prepubertally							
Monkey	Male	Juvenile	SP peptide	Castrated and GnRH primed	Bolus i.v.	No effect	Kalil <i>et al.</i> (2015)
Mouse	Female	Prepubertal	GR73632 (NK1R-Agonist)	Intact	i.c.v.	Stimulation	Simavli <i>et al.</i> (2015)
Rat	Female	Prepubertal	GR73632 (NK1R-Agonist)	Intact	i.c.v.	Stimulation	Ruiz-Pino <i>et al.</i> (2015)
Rat	Male	Prepubertal	GR73632 (NK1R-Agonist)	Intact	i.c.v.	Stimulation	Ruiz-Pino <i>et al.</i> (2015)

OVX, ovariectomized female; OVX+E₂, ovariectomized and estradiol treated female.

divergence in results using the NK1R antagonist (RPR 100893) in OVX + E₂-treated vs intact cycling monkeys. In the first study, the NK1R antagonist was administered in OVX + E₂-treated monkeys causing a 50% enhancement of the LH surge (Kerdelhue *et al.* 1997), supporting an inhibitory role of SP in the LH surge mechanism, similar to what was observed in the rat (Duval *et al.* 1996). By contrast, the same antagonist administered during the ascending phase of plasma estradiol concentrations (prior to LH surge onset of cycling monkeys), resulted in a reduction in both the amplitude (41%) and the duration of the preovulatory LH surge (Kerdelhue *et al.* 2000), providing evidence for a stimulatory role of SP in this model. Additional detailed analysis of changes in plasma SP concentration during the periovulatory period in women showed higher SP values during the day of the LH peak, the day of the descending phase and the day after the descending phase compared to all other stages in the menstrual cycle (Kerdelhue *et al.* 2006). However, a similar study carried out in the cycling monkey showed a decrease of plasma SP concentrations during the follicular phase leading to the LH surge and an inverse relationship between SP and estradiol values during this time (Kerdelhue *et al.* 2000). Thus, there appears to be a dual role for SP regarding the LH surge mechanism, as there have been inhibitory and stimulatory effects reported depending on species, sex steroid concentrations as well as the timing of exposure relative to the LH surge onset. The mechanism by which SP plays a role in the events leading up to the LH surge is not clear; however, the fact that ~25% of Kiss1 neurons in the AVPV/PeN contain *Tac1r* provides some input on the potential involvement of SP in this process (Navarro *et al.* 2015). In support of this notion is the observation that SP stimulates LH to a greater extent in female compared to male mice (Navarro *et al.* 2015), which are devoid of an AVPV/PeV Kiss1 population (Clarkson & Herbison 2006, Kauffman *et al.* 2007).

Precedent studies on the role of SP have also reported a potential action of SP on sexual behavior. The circuitry necessary for the expression of female sexual behavior,

and specifically the estrogen-induced display of lordosis, originates from the ventro-lateral VMN (vl VMN) and projects to the midbrain periaqueductal central gray (Pfaff & Sakuma 1979, Muntz *et al.* 1980, Yamanouchi *et al.* 1990). A number of studies have suggested that SP may be an important participant in this circuitry, as SP injections in the periaqueductal central gray of OVX, estrogen-primed rats produced a long-lasting increase of lordosis behavior (Dornan *et al.* 1987), whereas SP antiserum injections in the same region inhibit the behavior (Dornan *et al.* 1987). Interestingly, Fluoro-Gold injections into the dorsal midbrain labeled a large proportion (approximately 30%) of the vl VMN neurons immunoreactive for SP in the guinea pig (Ricciardi & Blaustein 1994). Furthermore, pulsatile administration of estradiol selectively induces the expression of progesterone receptors in SP neurons located in this area (Olster & Blaustein 1992), and this process is necessary for the induction of lordosis (Rubin & Barfield 1983). Collectively, these results suggest that SP originating in the vl VMN may participate in the onset of lordosis behavior (Dornan *et al.* 1990), however, further detailed components of the anatomy and physiology of this neurocircuitry is missing.

Neurokinin A

By contrast, much less information is available on the other members of the tachykinin family such as NKA or its two elongated peptides, NPK and NPy. NKA is also encoded by the *Tac1* gene in the rodent and preferentially binds to the NK2R (Beaujouan *et al.* 2000). The NKA/NK2R signaling system appears to act through different regulatory mechanisms, than those identified for SP; however, it is noteworthy that results to date have been a lot more consistent across species (Table 3). Central administration of the NK2R agonist, GR64349, displayed a NKB-like action in terms of LH release (the so called dual effect of senktide), showing inhibition in OVX mice but clear stimulation in OVX + E₂-treated female and intact male mice (Navarro *et al.* 2015). However, this

Table 3 Summary of results from *in vivo* studies investigating the effects of Neurokinin A on LH secretion in various species.

Species	Sex	Age	Drug	Gonadal status	Route	LH	Reference
With the presence of sex steroids							
Rat	Male	Adult	NKA peptide	Intact	i.c.v.	Stimulation	Kalra <i>et al.</i> (1992)
Mouse	Male	Adult	GR64349 (NK2R-Agonist)	Intact	i.c.v.	Stimulation	Navarro <i>et al.</i> (2015)
Mouse	Female	Adult	GR64349 (NK2R-Agonist)	OVX + E ₂	i.c.v.	Stimulation	Navarro <i>et al.</i> (2015)
Rat	Female	Adult	GR64349 (NK2R-Agonist)	Intact	i.c.v.	No effect	Ruiz-Pino <i>et al.</i> (2015)
Rat	Male	Adult	GR64349 (NK2R-Agonist)	Intact	i.c.v.	Stimulation	Ruiz-Pino <i>et al.</i> (2015)
Without the presence of sex steroids							
Rat	Male	Adult	NKA peptide	Castrated	i.c.v.	Inhibition	Kalra <i>et al.</i> (1992)
Rat	Female	Adult	NKA peptide	OVX	i.c.v.	No effect	Sahu and Kalra (1992)
Rat	Female	Adult	(Nle10)NKA 4–10 (NK2R-Agonist)	OVX	i.c.v.	Inhibition	Sahu and Kalra (1992)
Mouse	Female	Adult	GR64349 (NK2R-Agonist)	OVX	i.c.v.	Inhibition	Navarro <i>et al.</i> (2015)
Prepubertally							
Rat	Female	Prepubertal	GR64349 (NK2R-Agonist)	Intact	i.c.v.	Stimulation	Ruiz-Pino <i>et al.</i> (2015)
Rat	Male	Prepubertal	GR64349 (NK2R-Agonist)	Intact	i.c.v.	Stimulation	Ruiz-Pino <i>et al.</i> (2015)

OVX, ovariectomized female; OVX + E₂, ovariectomized and estradiol treated female.

stimulation does not take place in ovary-intact female rats (Ruiz-Pino *et al.* 2015), suggesting the existence of species differences. Overall, these data indicate that NK2R and NK3R may converge on a common pathway to regulate GnRH release in a sex-independent but sex steroid-dependent manner making them ideal candidates to participate in the GnRH pulse generator (Table 3). In this aspect, pharmacological studies in goats (Yamamura *et al.* 2015) and sheep (Fergani *et al.* 2016) showed that the three NKR agonists possess the ability to induce MUA volleys and an increase in LH respectively, albeit, with a significant difference in the efficacy to do so, as much higher concentrations of NK1R and NK2R agonists were required to have a similar effect as NKB agonist or senktide respectively (Fergani *et al.* 2016, Yamamura *et al.* 2015). Therefore, a reasonable hypothesis could be that NKA (and potentially SP) participates in the pulse generator by amplifying the actions of NKB. However, this requires further investigation as equivalent pulse studies are lacking in other species. Similar to what was previously suggested for the inhibitory action of NKB, the inhibitory action of NKA on LH release appears to also be opioid mediated, at least in the rat (Kalra *et al.* 1992). It is plausible to speculate that there is a sex steroid-dependent differential activation of the stimulatory (NK3R) or inhibitory receptor (KOR) after the administration of an NKA agonist in the presence vs absence of sex steroids; however, this remains to be proven.

Tachykinins modulate the gonadotropic axis in a kisspeptin-dependent manner

It is now well recognized that the stimulating effects of NKB on GnRH secretion are mediated primarily via initial kisspeptin stimulation. This has been demonstrated by studies that have shown that (a) desensitization of the kisspeptin receptor blocks the stimulatory effect of senktide in monkeys (Ramaswamy *et al.* 2011), (b) senktide i.c.v. administration induces c-Fos activation of kisspeptin cells in the ARC of rats (Navarro *et al.* 2011a), (c) as mentioned previously, nearly all ARC kisspeptin cells contain NK3R receptors (Navarro *et al.* 2009) and are excited by senktide/NKB (de Croft *et al.* 2013), (d) the stimulatory effect of senktide is completely absent in *Kiss1r*KO mice (Garcia-Galiano *et al.* 2012) and (e) specific ablation of NK3R expressing neurons in the ARC of the rat impairs the postcastration rise in LH secretion (Mittelman-Smith *et al.* 2012). The previously mentioned studies clearly indicate the importance of NKB signaling on kisspeptin for GnRH stimulation. However, additional regulation of GnRH release at a different level, i.e. kisspeptin-independent action, cannot be excluded given the presence of NK1R and NK3R in a subset of GnRH neurons (Krajewski *et al.* 2005, Navarro *et al.* 2015) and the reported kisspeptin-independent activation of GnRH neurons by NK3R agonists *in vitro* (Gaskins *et al.* 2013).

In this regard, a similar mechanism of action appears to be used by SP and NKA. Recent electrophysiological studies in a kisspeptin-green fluorescent protein mouse model have described potent stimulatory actions of SP and NKA on ARC Kiss1 neurons (de Croft *et al.* 2013). In addition, the administration of all individual tachykinin receptor agonists to mice lacking Kiss1r (*Kiss1r*KO mice) resulted in absent LH responses (Navarro *et al.* 2015). This, taken together with the fact that 50% of KNDy neurons contain NK1R (Navarro *et al.* 2015), suggests that SP is able to stimulate LH secretion by acting, at least in part, via a kisspeptin-dependent mechanism (Fig. 1). Intriguingly, in a recent study on female mice, NK1R agonist (GR73632) elicited a greater LH response than that observed with an NK2R agonist (GR64349; (Navarro *et al.* 2015)). It is possible that the augmented stimulatory action of NK1R agonist on LH release is a reflection of the additional action of SP on both populations of Kiss1 neurons (ARC and AVPV/PeN) (Navarro *et al.* 2015). In support of this hypothesis, the same exaggerated effect of NK1R agonist was not observed in male mice (Navarro *et al.* 2015), which also lack an AVPV kiss1 neuronal population (Smith *et al.* 2005, Kauffman *et al.* 2007). Potential direct action on GnRH neurons, however, cannot be overlooked, as at least in the mouse, a subset of GnRH neurons express SP (and NKB) receptors (Navarro *et al.* 2015) and senktide can induce *in vitro* GnRH secretion in the ME in brain slices derived from *Kiss1*-knockout mice (Gaskins *et al.* 2013). In this light, a very important question arises, which is also true for the action of NKB, as to which pathway is used when (kisspeptin vs GnRH dependent pathways) and for what biological purpose. Potentially, as the majority of studies investigating the necessity of an intact Kiss1/Kiss1r signaling system in the stimulation of LH secretion by tachykinins have been carried out in the persistent hypogonadal state (primarily via the blockade of kiss1r; see above), it is plausible to speculate that the sex steroid milieu may be an important determining factor. Studies carried out with or without the presence of sex steroids and an absent Kiss1/Kiss1r system may be useful in this aspect. The action of NKA, however, is less clear, because *Tacr2* is not present in either Kiss1 or GnRH neurons, while showing a kisspeptin-dependent action (Navarro *et al.* 2015), thus suggesting the presence of unidentified intermediate neurons upstream of Kiss1 neurons. Nonetheless, even though there are still major gaps in our knowledge regarding the potential mechanisms used by each tachykinin, current data are overall, placing tachykinins in the spotlight as prime candidates for the neuromodulation of kisspeptin release.

Despite substantial evidence for the hypothalamic action of tachykinins, we cannot ignore observations that suggest a direct action of SP and NKA in the pituitary. Firstly, SP fibers have been observed to surround hypophyseal portal blood capillary vessels in the ME in

monkeys (Kalil *et al.* 2015) and NKRs have been shown to exist in pituitary cells in rats (Larsen *et al.* 1992) and sheep (Dupre *et al.* 2010). Second, it has been reported that SP and NKA can stimulate LH secretion from cultured anterior pituitary cells derived from intact male rats (Kalra *et al.* 1992) and hemi-pituitaries (Shamgochian & Leeman 1992) respectively. Furthermore, in the pig, this has been shown to be a result of direct action upon gonadotropes (Hidalgo-Díaz *et al.* 1998), and depends on extracellular and intracellular Ca^{2+} levels, but does not involve net increases in LH β mRNA levels (Hidalgo-Díaz *et al.* 2003). These findings, however, are not consistent as the same was not observed in dispersed anterior pituitary cells harvested from female OVX+E₂ rats (Arisawa *et al.* 1990). Clearly, this pathway of action requires further investigation. For example, it would be interesting to evaluate whether LH secretion is stimulated after the peripheral administration of NKR agonists, but in the presence of a GnRH antagonist, to rule out any central effects on, or above, GnRH neurons that these agonists might exert by crossing the blood–brain barrier. This approach could potentially shed more light on the likelihood of a pituitary action of tachykinins.

The role of tachykinins on puberty onset

The precise neuronal and endocrine mechanisms that determine the timing of puberty onset, and the subsequent achievement of reproductive capacity, remains one of the greatest unanswered questions in reproductive biology. To date, several factors from central and peripheral origins have been described to regulate the awakening of the gonadotropic axis (Ojeda & Lomniczi 2014). At a neuroendocrine level, the prevailing view is that during the infantile and juvenile periods, neurons secreting GnRH are subjected to persistent synaptic inhibition (Ojeda *et al.* 2010). When this inhibition is removed, GnRH secretion increases, which leads to puberty. However, it is recognized that a gain in numerous excitatory inputs to GnRH neurons is also indispensable (Ojeda & Lomniczi 2014). In this respect, both loss-of-function and gain-of-function mutations in a growing number of neurotransmitters, and their receptors have been described to severely impinge on the pubertal transition. As mentioned previously, a number of studies have documented lack or delay of pubertal maturation in humans and mice bearing loss-of-function mutations in *KISS1/KISS1R* or *TAC3/TACR3* genes (de Roux *et al.* 2003, Seminara *et al.* 2003, Young *et al.* 2010, Topaloglu *et al.* 2012). In contrast, gain-of-function mutations in *KISS1R* have been identified in association with central precocious puberty (Teles *et al.* 2008). Therefore, kisspeptins are indispensable regulatory signals of GnRH release during puberty (Seminara *et al.* 2003). In the same vein, the tachykinin NKB has been reported to stimulate kisspeptin prepubertally (Navarro *et al.* 2012) and the expression of *Tac2* increases before *Kiss1*

(Gill *et al.* 2012), suggesting a likely role of this tachykinin in the pubertal activation of kisspeptin–GnRH secretion (Topaloglu *et al.* 2009, Young *et al.* 2010).

The equivalent role of SP and NKA in the prepubertal increase of LH release and their contribution to the timing of puberty onset has only recently begun to draw attention. A series of functional tests and genetic studies in the female mouse have shown that SP/NK1R and NKA/NK2R signaling appears to participate in the timing of puberty. This conclusion is derived from a study by Simavli and coworkers (2015), which has shown that (1) a selective NK1R agonist induces LH release in prepubertal females; (2) the expression of *Tac1* and *Tacr1* in the ARC is increased just before puberty compared with earlier or later stages of postnatal development; (3) repeated exposure to NK1R agonists prepubertally advances puberty onset, suggesting that the NK1R is already present and functional during this developmental period. Furthermore, (4) *Tac1*KO female mice exhibit a significant delay in vaginal opening (defined as complete canalization of the vagina, an event that occurs with increased estrogen secretion (Caligioni 2009) and is therefore considered an indirect marker for puberty onset) and delayed initiation of estrous cyclicity (Simavli *et al.* 2015). This suggests that although E₂ is produced by the ovaries in these mice, this alone may not be sufficient to trigger an LH surge during the initial phase post-vaginal opening, and this positive feedback may also be compromised during adulthood. Indeed, histological examination of the ovaries revealed fewer numbers of corpus lutea and antral follicles in *Tac1*-knockout mice. Similarly, in the rat, administration of NK1R and NK2R agonists was able to significantly increase LH release in prepubertal animals of both sexes, with NK2R agonist evoking a significantly greater response than that by NK1R agonist in both males and females (Ruiz-Pino *et al.* 2015). By contrast, castrated, juvenile and GnRH primed monkeys did not respond to an i.v. bolus administration of SP with an increase in LH secretion (Kalil *et al.* 2015). The reason for this is not known; however, it may reflect a species difference. Interestingly, supporting the role of SP in the central control of puberty onset is the fact that higher SP levels detected in the brain of patients after traumatic brain injury (Vink & van den Heuvel 2010, Zacest *et al.* 2010, Gabrielian *et al.* 2013) correlate with the significantly higher ratio of children displaying precocious puberty after traumatic brain injury (Blendonohy & Philip 1991, Kaulfers *et al.* 2010). Overall, these data suggest a greater sensitivity to hypothalamic SP (and possibly NKA), at the time of puberty initiation, presumably contributing to an increase in GnRH pulses and activation of the gonadotropic axis; however, despite the compelling evidence for a central role of SP, we cannot rule out the possibility of actions of SP in other organs of the gonadotropic axis, such as the ovary (Debeljuk 2003, 2006).

Concluding remarks

Elucidating the neuronal mechanisms generating the GnRH pulses and surge is a prerequisite in advancing our understanding of reproductive function. This review intends to discuss the existing literature on the role of tachykinins as important components of this mechanism leading to GnRH and, therefore, LH secretion (model hypothesis; Fig. 1). Overall, substantial evidence exists to support the hypothesis that tachykinins are indeed involved in the control of GnRH release, by modulating the firing of ARC KNDy neurons either directly (NKB and SP) or indirectly (NKA) to shape kisspeptin pulses (Fig. 1). In addition, tachykinins, particularly SP may also act directly on GnRH and/or AVPV/PeN Kiss1 neurons to contribute to: (a) the shaping of GnRH pulses, and/or (b) the generation of the preovulatory LH surge. Many aspects of the physiology of the SP/NK1R, NKA/NK2R signaling systems in the context of reproduction, remain to be fully characterized. For instance, there appears to be a relative inconsistency in results between mice, rats, ruminants and monkeys in the LH response to the administration of tachykinins that may reflect anatomical and functional differences among species. In this regard, in humans SP is colocalized within a subset of KNDy neurons (Hrabovszky *et al.* 2013), whereas this is not true for all other species studied to date (Rance & Young 1991, Rance & Bruce 1994, Kalil *et al.* 2015, Navarro *et al.* 2015). Furthermore, in ruminants, a much larger dose of SP is required to stimulate LH release to a similar magnitude as an NKB agonist (Fergani *et al.* 2016, Yamamura *et al.* 2015), whereas in mice, similar doses of all individual NKR agonists can lead to an increase in LH (Navarro *et al.* 2015). However, as discussed, routes of administration, age (prepubertal vs postpubertal) and sex steroid status might be a determining factor in this aspect and must be taken in to account. Another important parameter that requires specific attention in future studies is the considerable cross-reactivity that exists between these receptor/ligand systems determining the efficacy of tachykinin administration and it may be that although the three NKRs are involved in the GnRH pulse generation of KNDy neurons, the ratio of the contribution of each NKR varies among species and/or sexes. Nonetheless, this phenomenon may offer important advantages in the treatment of disorders caused by disruption of one specific system. For example, the reversal phenotype in reproductive viability observed in individuals with *TAC3/TACR3* mutations (Gianetti *et al.* 2010) may be due to compensation by the other tachykinin systems although this remains to be elucidated. Altogether, there is a clear need for a deeper understanding of the mechanism of action of tachykinins. We must answer: (a) whether all tachykinins participate in the generation of LH pulses, (b) if there is compensation between tachykinins to exert this role and to what extent, (c) whether the pathway

(KNDy vs GnRH) of tachykinin action is governed by sex steroid levels and the biological role of this interaction, (d) if the expression of tachykinin receptors in GnRH neurons changes (increases or decreases) in an estradiol dependent manner, (e) the anatomical relationship of tachykinins and their receptors with kisspeptin and GnRH perikarya and fibers in other species, apart from the mouse, (f) the sex and species differences in the response to tachykinins and the contribution of SP/NK1R signaling on AVPV/PeN Kiss1 neurons or GnRH for the occurrence of the GnRH/LH surge in the female, (h) the mechanism and site of action of NKA, as well as the phenotype of the cells that contain NK2R, which appear to be surrogates for the indirect action of Tac1 on KNDy neurons.

All of these unresolved questions are fundamental to the understanding the mechanisms that govern GnRH release in mammals, and the outcome of studies such as these may prompt a change in the thinking of the current models of GnRH pulse generation. Moreover, expanding the current model will have tremendous clinical potential in humans as there is a large number of disorders associated with dysregulation of GnRH release, e.g. delayed and precocious puberty, polycystic ovarian syndrome, hormone-dependent tumors, that could be treated in a more physiological and effective manner.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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