Expanding the Role of Tachykinins in the Neuroendocrine Control of Reproduction.

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Contents

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), so called KNDy neurons, release kisspeptin (a potent GnRH secretagogue) in a pulsatile manner to drive GnRH pulses under the coordinated autosynaptic 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.

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-gonad (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 (Herbison 2016, Herbison, et al. 2008, Moenter, et al. 2003). 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; (Hrabovszky and Liposits 2013, Radovick, et al. 2012, 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 in to the hypophyseal portal vessels, thereby enabling gonadotropin [luteinizing hormone (LH) and follicle stimulating hormone (FSH)] secretion from the anterior pituitary in to 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 (Chan, et al. 2011, de Roux, et al. 2003, Seminara, et al. 2003, 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 regulatory inputs that modulate kisspeptin secretion for the initiation of puberty and the maintenance of fertility in adulthood (Pinilla, et al. 2012, Seminara, et al. 2003). Importantly, Kiss1 neurons also play a critical role in conveying information about the sex steroid milieu to GnRH neurons (Gill, et al. 2010, Navarro,

et al. 2004). 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 comprised by the tachykinin neurokinin B (NKB) and its receptor (NK3R), encoded by *TAC3* and *TACR3* in humans, respectively (Table 1). This system has received substantial attention since 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, Topaloglu, et al. 2012, Yang, et al. 2012, Young, et al. 2010), 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, Fraser, et al. 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 (Goodman, et al. 2014, Navarro 2013) 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 since 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 and Navarro 2012, True, et al. 2015, Yang, et al. 2012). 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-NH_2 (Maggio 1988). This family also includes substance P (SP), neurokinin A (NKA), neuropeptide K (NPK), and neuropeptide γ (NPγ) (Otsuka and 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 and 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 and 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 (Beaujouan, et al. 2000, Cascieri, et al. 1992, Gether, et al. 1993). In these studies, the affinities or EC50 values of each tachykinin for NK1R, NK2R, and NK3R, respectively, were reported as follows: SP_2nM, 2200nM, and 18000nM; NKA _ 16nM, 3nM, and 1300nM; and NKB_70nM, 25nM, and 4nM (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 and 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 mediate the positive feedback that leads to the female-specific preovulatory GnRH/LH surge (Maeda, et al. 2007, Navarro, et al. 2004, Smith, et al. 2005). 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, Goodman, et al. 2013, Navarro 2012), 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 autosynaptic 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 (Amstalden, et al. 2010, Navarro, et al. 2009, Navarro, et al. 2011b) 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 (Krajewski, et al. 2010, Lehman, et al. 2010, Rance and Bruce 1994). 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 (Goodman, et al. 2014, Grachev, et al. 2012, Navarro 2013). 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, de Croft, et al. 2013, Kalra, et al. 1992, Navarro, et al. 2015, Noritake, et al. 2011, Sahu and Kalra 1992) 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 (Goubillon, et al. 2000, Navarro, et al. 2009, Rance and Young 1991). 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 (Borsay, et al. 2014, Harlan, et al. 1989, Rance and Bruce 1994, Rance

and Young 1991, Ronnekleiv, et al. 1984, Tsuruo, et al. 1991, Yamano, et al. 1986). Studies employing 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) 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, Hrabovszky 2014, Navarro, et al. 2009) the *Tac1*-positive neurons did not colocalize with *Kiss1*-positive neurons in the mouse [(Navarro, et al. 2015); Figure.1). This is in agreement with equivalent investigations in the monkey (Kalil, et al. 2015) and rat (Rance and Bruce 1994) but contradict findings in the human that report 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 (Figure. 1), the region in which the second population of Kiss1 neurons reside (Oakley, et al. 2009), however, data from other species is 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)]. *Tacr2*, however, was absent from both populations of Kiss1 neurons and GnRH neurons (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, as has been previously described in various species (Amstalden, et al. 2010, Navarro, et al. 2015, Navarro, et al. 2009). 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). 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). 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 indirectly, via 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); Figure.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 *Tac1*-expressing neurons in the ARC and VMN of mice were downregulated by OVX and E_2 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- compared to pre-menopausal women (Rance and 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 and 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 versus 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 (Navarro, et al. 2011a, Ruiz-Pino, et al. 2012, Sandoval-Guzman and Rance 2004). 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 (Grachev, et al. 2012, Navarro, et al. 2015, Navarro, et al. 2011b), 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 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 and Singewald 2006). Even though SP was originally identified in the 1930's (Lasaga and 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+E_2 rats, stimulated LH release, via both routes of administration (Arisawa, et al. 1990). Other studies conducted by Kalra et al., in the 90's (Kalra, et al. 1992) (Sahu and Kalra 1992) report null or inhibitory effects in intact and GND males, respectively, hinting at potential sex differences in the 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 towards 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+E_2 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 senktide (Goodman 2015, Yamamura, et al. 2015).

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 et al., 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 divergence in results using the NK1R antagonist (RPR 100893) in OVX + E_2 treated *versus* intact cycling monkeys. In the first study, the NK1R antagonist was administered in OVX + E_2 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 up 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 a 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 and 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 (Muntz, et al. 1980, Pfaff and Sakuma 1979, 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 and Blaustein 1994)**.** Furthermore, pulsatile administration of estradiol, selectively induces the expression of

progesterone receptors in SP neurons located in this area (Olster and Blaustein 1992) and this process is necessary for the induction of lordosis (Rubin and 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 NPγ. 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+E_2 treated female and intact male mice (Navarro, et al. 2015). Similar results have been obtained by studies conducted in male and female rats (Kalra, et al. 1992, Ruiz-Pino, et al. 2015, Sahu and Kalra 1992). 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 (Goodman 2015), 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 (Goodman 2015, Yamamura, et al. 2015). Therefore, a reasonable hypothesis could be that NKA (and potentially SP) participate 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 versus 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 above, 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 above 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 employed 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 (Figure. 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 (Kauffman, et al. 2007, Smith, et al. 2005). 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 employed when (kisspeptin versus 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 employed 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 NKR's 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 and Leeman 1992), respectively. These findings however, are not consistent as the same was not observed in dispersed anterior pituitary cells harvested from female OVX+E_2 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 and 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 and 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 above, 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, Topaloglu, et al. 2012, Young, et al. 2010). 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 began 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, et al. 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 to 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

485 an indirect maker for puberty onset] and delayed initiation of estrous cyclicity (Simavli, et al. 2015).
486 This suggests that although E_2 is produced by the ovaries in these mice, this alone may not be
487 sufficient to trigger an LH surge during the initial phase post vaginal opening and this positive
488 feedback may also be compromised during adulthood. Indeed, histological examination of the
489 ovaries revealed fewer numbers of corpus lutea and antral follicles in *Tac1* knockout mice.
490 Similarly, in the rat, administration of NK1R and NK2R agonists was able to significantly increase
491 LH release in prepubertal animals of both sexes, with NK2R agonist evoking a significantly greater
492 response than that by NK1R agonist in both males and females (Ruiz-Pino, et al. 2015). By
493 contrast castrated, juvenile and GnRH primed monkeys did not respond to an i.v. bolus
494 administration of SP with an increase in LH secretion (Kalil, et al. 2015). The reason for this is not
495 known however it may reflect a species difference. Interestingly, supporting the role of SP in the
496 central control of puberty onset is the fact that higher SP levels detected in the brain of patients
497 after traumatic brain injury (Gabrielian, et al. 2013, Vink and van den Heuvel 2010, Zacest, et al.
498 2010) correlate with the significantly higher ratio of children displaying precocious puberty after
499 traumatic brain injury (Blendonohy and Philip 1991, Kaulfers, et al. 2010). Overall, these data
500 suggest a greater sensitivity to hypothalamic SP (and possibly NKA), at the time of puberty
501 initiation, presumably contributing to an increase in GnRH pulses and activation of the
502 gonadotropic axis; however, despite the compelling evidence for a central role of SP, we cannot
503 rule out the possibility of actions of SP in other organs of the gonadotropic axis, such as the ovary
504 (Debeljuk 2003, 2006).

505 Concluding remarks

506 Elucidating the neuronal mechanisms generating the GnRH pulses and surge is a prerequisite in
507 advancing our understanding of reproductive function. This review intends to discuss the existing
508 literature on the role of tachykinins as important components of this mechanism leading to GnRH

and therefore, LH secretion (model hypothesis; Figure 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 (Figure 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 (Kalil, et al. 2015, Navarro, et al. 2015, Rance and Bruce 1994, Rance and Young 1991). Furthermore, in ruminants, a much larger dose of SP is required to stimulate LH release to a similar magnitude as an NKB agonist (Goodman 2015, 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 versus 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 crossreactivity 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 versus 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 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, since 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.

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Figure legends.

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.

References.

Akesson, TR, and PE Micevych 1988 Estrogen concentration by substance P-immunoreactive neurons in the medial basal hypothalamus of the female rat. *J Neurosci Res* **19** 412-419, 470-411.

Amstalden, M, LM Coolen, AM Hemmerle, HJ Billings, JM Connors, RL Goodman, and MN Lehman 2010 Neurokinin 3 receptor immunoreactivity in the septal region, preoptic area and hypothalamus of the female sheep: colocalisation in neurokinin B cells of the arcuate nucleus but not in gonadotrophin-releasing hormone neurones. *J Neuroendocrinol* **22** 1-12.

Arisawa, M, L De Palatis, R Ho, GD Snyder, WH Yu, G Pan, and SM McCann 1990 Stimulatory role of substance P on gonadotropin release in ovariectomized rats. *Neuroendocrinology* **51** 523-529.

Beaujouan, JC, M Saffroy, Y Torrens, and J Glowinski 2000 Different subtypes of tachykinin NK(1) receptor binding sites are present in the rat brain. *J Neurochem* **75** 1015-1026.

Billings, HJ, JM Connors, SN Altman, SM Hileman, I Holaskova, MN Lehman, CJ McManus, CC Nestor, BH Jacobs, and RL Goodman 2010 Neurokinin B acts via the neurokinin-3 receptor in the retrochiasmatic area to stimulate luteinizing hormone secretion in sheep. *Endocrinology* **151** 3836-3846.

Blendonohy, PM, and PA Philip 1991 Precocious puberty in children after traumatic brain injury. *Brain Inj* **5** 63-68.

Borsay, BA, K Skrapits, L Herczeg, P Ciofi, SR Bloom, MA Ghatei, WS Dhillo, Z Liposits, and E Hrabovszky 2014 Hypophysiotropic gonadotropin-releasing hormone projections are exposed to dense plexuses of kisspeptin, neurokinin B and substance p immunoreactive fibers in the human: a study on tissues from postmenopausal women. *Neuroendocrinology* **100** 141-152.

Caligioni, CS 2009 Assessing reproductive status/stages in mice. *Curr Protoc Neurosci* **Appendix 4** Appendix 4I.

Cascieri, MA, RR Huang, TM Fong, AH Cheung, S Sadowski, E Ber, and CD Strader 1992 Determination of the amino acid residues in substance P conferring selectivity and specificity for the rat neurokinin receptors. *Mol Pharmacol* **41** 1096-1099.

Chan, YM, S Broder-Fingert, S Paraschos, R Lapatto, M Au, V Hughes, SD Bianco, L Min, L Plummer, F Cerrato, A De Guillebon, IH Wu, F Wahab, A Dwyer, S Kirsch, R Quinton, T Cheetham, M Ozata, S Ten, JP Chanoine, N Pitteloud, KA Martin, R Schiffmann, HJ Van der Kamp, S Nader, JE Hall, UB Kaiser, and SB Seminara 2011 GnRH-deficient phenotypes in humans and mice with heterozygous variants in KISS1/Kiss1. *J Clin Endocrinol Metab* **96** E1771-1781.

Cheng, G, LM Coolen, V Padmanabhan, RL Goodman, and MN Lehman 2010 The kisspeptin/neurokinin B/dynorphin (KNDy) cell population of the arcuate nucleus: sex differences and effects of prenatal testosterone in sheep. *Endocrinology* **151** 301-311.

Clarkson, J, X d'Anglemont de Tassigny, WH Colledge, A Caraty, and AE Herbison 2009 Distribution of kisspeptin neurones in the adult female mouse brain. *J Neuroendocrinol* **21** 673-682.

Clarkson, J, and AE Herbison 2006 Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons. *Endocrinology* **147** 5817-5825.

Coiro, V, R Volpi, L Capretti, A Caiazza, A Marcato, R Bocchi, R Colla, G Rossi, and P Chiodera 1992 Luteinizing hormone response to an intravenous infusion of substance P in normal men. *Metabolism* **41** 689-691.

de Croft, S, U Boehm, and AE Herbison 2013 Neurokinin B activates arcuate kisspeptin neurons through multiple tachykinin receptors in the male mouse. *Endocrinology* **154** 2750-2760.

De Felipe, C, JF Herrero, JA O'Brien, JA Palmer, CA Doyle, AJ Smith, JM Laird, C Belmonte, F Cervero, and SP Hunt 1998 Altered nociception, analgesia and aggression in mice lacking the receptor for substance P. *Nature* **392** 394-397.

de Roux, N, E Genin, JC Carel, F Matsuda, JL Chaussain, and E Milgrom 2003 Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc Natl Acad Sci U S A* **100** 10972-10976.

Debeljuk, L 2003 Tachykinins in the normal and gonadotropin-stimulated ovary of the mouse. *Peptides* **24** 1445-1448.

Debeljuk, L 2006 Tachykinins and ovarian function in mammals. *Peptides* **27** 736-742.

Dobson, H, S Ghuman, S Prabhakar, and R Smith 2003 A conceptual model of the influence of stress on female reproduction. *Reproduction* **125** 151-163.

Dornan, WA, TR Akesson, and PE Micevych 1990 A substance P projection from the VMH to the dorsal midbrain central gray: implication for lordosis. *Brain Res Bull* **25** 791-796.
Dornan, WA, CW Malsbury, and RB Penney 1987 Facilitation of lordosis by injection of substance P into the midbrain central gray. *Neuroendocrinology* **45** 498-506.
Dupre, SM, K Miedzinska, CV Duval, L Yu, RL Goodman, GA Lincoln, JR Davis, AS McNeilly, DD Burt, and AS Loudon 2010 Identification of Eya3 and TAC1 as long-day signals in the sheep pituitary. *Curr Biol* **20** 829-835.
Duval, P, V Lenoir, C Garret, and B Kerdelhue 1996 Reduction of the amplitude of preovulatory LH and FSH surges and of the amplitude of the in vitro GnRH-induced LH release by substance P. Reversal of the effect by RP 67580. *Neuropharmacology* **35** 1805-1810.
Ebner, K, and N Singewald 2006 The role of substance P in stress and anxiety responses. *Amino Acids* **31** 251-272.
Fraser, GL, HR Hoveyda, IJ Clarke, S Ramaswamy, TM Plant, C Rose, and RP Millar 2015 The NK3 Receptor Antagonist ESN364 Interrupts Pulsatile LH Secretion and Moderates Levels of Ovarian Hormones Throughout the Menstrual Cycle. *Endocrinology* **156** 4214-4225.
Fraser, GL, S Ramael, HR Hoveyda, L Gheyle, and J Combalbert 2016 The NK3 Receptor Antagonist ESN364 Suppresses Sex Hormones in Men and Women. *J Clin Endocrinol Metab* **101** 417-426.
Gabrielian, L, SC Helps, E Thornton, RJ Turner, AV Leonard, and R Vink 2013 Substance P antagonists as a novel intervention for brain edema and raised intracranial pressure. *Acta Neurochir Suppl* **118** 201-204.
Garcia-Galiano, D, D van Ingen Schenau, S Leon, MA Krajnc-Franken, M Manfredi-Lozano, A Romero-Ruiz, VM Navarro, F Gaytan, PI van Noort, L Pinilla, M Blomenrohr, and M Tena-Sempere 2012 Kisspeptin signaling is indispensable for neurokinin B, but not glutamate, stimulation of gonadotropin secretion in mice. *Endocrinology* **153** 316-328.
Gaskins, GT, KM Glanowska, and SM Moenter 2013 Activation of neurokinin 3 receptors stimulates GnRH release in a location-dependent but kisspeptin-independent manner in adult mice. *Endocrinology* **154** 3984-3989.
Gether, U, TE Johansen, and TW Schwartz 1993 Chimeric NK1 (substance P)/NK3 (neurokinin B) receptors. Identification of domains determining the binding specificity of tachykinin agonists. *J Biol Chem* **268** 7893-7898.
Gianetti, E, C Tusset, SD Noel, MG Au, AA Dwyer, VA Hughes, AP Abreu, J Carroll, E Trarbach, LF Silveira, EM Costa, BB de Mendonca, M de Castro, A Lofrano, JE Hall, E Bolu, M Ozata, R Quinton, JK Amory, SE Stewart, W Arlt, TR Cole, WF Crowley, UB Kaiser, AC Latronico, and SB Seminara 2010 TAC3/TACR3 mutations reveal preferential activation of gonadotropin-releasing hormone release by neurokinin B in neonatal life followed by reversal in adulthood. *J Clin Endocrinol Metab* **95** 2857-2867.
Gill, JC, VM Navarro, C Kwong, SD Noel, C Martin, S Xu, DK Clifton, RS Carroll, RA Steiner, and UB Kaiser 2012 Increased neurokinin B (Tac2) expression in the mouse arcuate nucleus is an early marker of pubertal onset with differential sensitivity to sex steroid-negative feedback than Kiss1. *Endocrinology* **153** 4883-4893.
Gill, JC, O Wang, S Kakar, E Martinelli, RS Carroll, and UB Kaiser 2010 Reproductive hormone-dependent and -independent contributions to developmental changes in kisspeptin in GnRH-deficient hypogonadal mice. *PLoS One* **5** e11911.
Goodman, RL, LM Coolen, and MN Lehman 2014 A role for neurokinin B in pulsatile GnRH secretion in the ewe. *Neuroendocrinology* **99** 18-32.
Goodman, RL, SM Hileman, CC Nestor, KL Porter, JM Connors, SL Hardy, RP Millar, M Cernea, LM Coolen, and MN Lehman 2013 Kisspeptin, neurokinin B, and dynorphin

act in the arcuate nucleus to control activity of the GnRH pulse generator in ewes. *Endocrinology* **154** 4259-4269.

Goodman, RL, MN Lehman, JT Smith, LM Coolen, CV de Oliveira, MR Jafarzadehshirazi, A Pereira, J Iqbal, A Caraty, P Ciofi, and IJ Clarke 2007 Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin A and neurokinin B. *Endocrinology* **148** 5752-5760.

Goodman, RLLMRBMPGJMCSMH 2015 High Doses of Neurokinin A and Substance P Stimulate LH Secretion in Ewes - See more at: http://press.endocrine.org/doi/abs/10.1210/endo-meetings.2015.np.3.fri-437#sthash.6n2dFX5K.dpuf, Endocrine Society's 97th Annual Meeting and Expo. San Diego, CA.

Gottsch, ML, VM Navarro, Z Zhao, C Glidewell-Kenney, J Weiss, JL Jameson, DK Clifton, JE Levine, and RA Steiner 2009 Regulation of Kiss1 and dynorphin gene expression in the murine brain by classical and nonclassical estrogen receptor pathways. *J Neurosci* **29** 9390-9395.

Goubillon, ML, RA Forsdike, JE Robinson, P Ciofi, A Caraty, and AE Herbison 2000 Identification of neurokinin B-expressing neurons as an highly estrogen-receptive, sexually dimorphic cell group in the ovine arcuate nucleus. *Endocrinology* **141** 4218-4225.

Grachev, P, XF Li, YS Lin, MH Hu, L Elsamani, SJ Paterson, RP Millar, SL Lightman, and KT O'Byrne 2012 GPR54-dependent stimulation of luteinizing hormone secretion by neurokinin B in prepubertal rats. *PLoS One* **7** e44344.

Harlan, RE, MM Garcia, and JE Krause 1989 Cellular localization of substance P- and neurokinin A-encoding preprotachykinin mRNA in the female rat brain. *J Comp Neurol* **287** 179-212.

Herbison, AE 1998 Multimodal influence of estrogen upon gonadotropin-releasing hormone neurons. *Endocr Rev* **19** 302-330.

Herbison, AE 2016 Control of puberty onset and fertility by gonadotropin-releasing hormone neurons. *Nat Rev Endocrinol* **12** 452-466.

Herbison, AE, R Porteous, JR Pape, JM Mora, and PR Hurst 2008 Gonadotropin-releasing hormone neuron requirements for puberty, ovulation, and fertility. *Endocrinology* **149** 597-604.

Hrabovszky, E 2014 Neuroanatomy of the human hypothalamic kisspeptin system. *Neuroendocrinology* **99** 33-48.

Hrabovszky, E, BA Borsay, K Racz, L Herczeg, P Ciofi, SR Bloom, MA Ghatei, WS Dhillo, and Z Liposits 2013 Substance p immunoreactivity exhibits frequent colocalization with kisspeptin and neurokinin B in the human infundibular region. *PLoS One* **8** e72369.

Hrabovszky, E, and Z Liposits 2013 Afferent neuronal control of type-I gonadotropin releasing hormone neurons in the human. *Front Endocrinol (Lausanne)* **4** 130.

Kalil, B, S Ramaswamy, and TM Plant 2015 The Distribution of Substance P and Kisspeptin in the Mediobasal Hypothalamus of The male Rhesus Monkey and a Comparison of Intravenous Administration of These Peptides to Release GnRH as Reflected by LH Secretion. *Neuroendocrinology*.

Kalra, PS, A Sahu, JJ Bonavera, and SP Kalra 1992 Diverse effects of tachykinins on luteinizing hormone release in male rats: mechanism of action. *Endocrinology* **131** 1195-1201.

Kauffman, AS, ML Gottsch, J Roa, AC Byquist, A Crown, DK Clifton, GE Hoffman, RA Steiner, and M Tena-Sempere 2007 Sexual differentiation of Kiss1 gene expression in the brain of the rat. *Endocrinology* **148** 1774-1783.

Kaulfers, AM, PF Backeljauw, K Reifschneider, S Blum, L Michaud, M Weiss, and SR Rose 2010 Endocrine dysfunction following traumatic brain injury in children. *J Pediatr* **157** 894-899.

Kawakami, M, T Uemura, and R Hayashi 1982 Electrophysiological correlates of pulsatile gonadotropin release in rats. *Neuroendocrinology* **35** 63-67.

Keen, KL, FH Wegner, SR Bloom, MA Ghatei, and E Terasawa 2008 An increase in kisspeptin-54 release occurs with the pubertal increase in luteinizing hormone-releasing hormone-1 release in the stalk-median eminence of female rhesus monkeys in vivo. *Endocrinology* **149** 4151-4157.

Kerdelhue, B, K Gordon, R Williams, V Lenoir, V Fardin, P Chevalier, C Garret, P Duval, P Kolm, G Hodgen, H Jones, and GS Jones 1997 Stimulatory effect of a specific substance P antagonist (RPR 100893) of the human NK1 receptor on the estradiol-induced LH and FSH surges in the ovariectomized cynomolgus monkey. *J Neurosci Res* **50** 94-103.

Kerdelhue, B, V Lenoir, R Scholler, and HW Jones, Jr. 2006 Substance P plasma concentration during the LH preovulatory surge of the menstrual cycle in the human. *Neuro Endocrinol Lett* **27** 359-364.

Kerdelhue, B, RF Williams, V Lenoir, V Fardin, P Kolm, GD Hodgen, GS Jones, R Scholler, and HW Jones, Jr. 2000 Variations in plasma levels of substance P and effects of a specific substance P antagonist of the NK(1) receptor on preovulatory LH and FSH surges and progesterone secretion in the cycling cynomolgus monkey. *Neuroendocrinology* **71** 228-236.

Kinsey-Jones, JS, P Grachev, XF Li, YS Lin, SR Milligan, SL Lightman, and KT O'Byrne 2012 The inhibitory effects of neurokinin B on GnRH pulse generator frequency in the female rat. *Endocrinology* **153** 307-315.

Krajewski, SJ, MJ Anderson, L Iles-Shih, KJ Chen, HF Urbanski, and NE Rance 2005 Morphologic evidence that neurokinin B modulates gonadotropin-releasing hormone secretion via neurokinin 3 receptors in the rat median eminence. *J Comp Neurol* **489** 372-386.

Krajewski, SJ, MC Burke, MJ Anderson, NT McMullen, and NE Rance 2010 Forebrain projections of arcuate neurokinin B neurons demonstrated by anterograde tract-tracing and monosodium glutamate lesions in the rat. *Neuroscience* **166** 680-697.

Larsen, PJ, T Saermark, and SE Mau 1992 Binding of an iodinated substance P analogue to cultured anterior pituitary prolactin- and luteinizing hormone-containing cells. *J Histochem Cytochem* **40** 487-493.

Lasaga, M, and L Debeljuk 2011 Tachykinins and the hypothalamo-pituitary-gonadal axis: An update. *Peptides* **32** 1972-1978.

Lehman, MN, LM Coolen, and RL Goodman 2010 Minireview: kisspeptin/neurokinin B/dynorphin (KNDy) cells of the arcuate nucleus: a central node in the control of gonadotropin-releasing hormone secretion. *Endocrinology* **151** 3479-3489.

Leon, S, A Barroso, MJ Vazquez, D Garcia-Galiano, M Manfredi-Lozano, F Ruiz-Pino, V Heras, A Romero-Ruiz, J Roa, G Schutz, M Kirilov, F Gaytan, L Pinilla, and M Tena-Sempere 2016 Direct Actions of Kisspeptins on GnRH Neurons Permit Attainment of Fertility but are Insufficient to Fully Preserve Gonadotropic Axis Activity. *Sci Rep* **6** 19206.

Luque, RM, J Cordoba-Chacon, MD Gahete, VM Navarro, M Tena-Sempere, RD Kineman, and JP Castano 2011 Kisspeptin regulates gonadotroph and somatotroph function in nonhuman primate pituitary via common and distinct signaling mechanisms. *Endocrinology* **152** 957-966.

Maeda, K, S Adachi, K Inoue, S Ohkura, and H Tsukamura 2007 Metastin/kisspeptin and control of estrous cycle in rats. *Rev Endocr Metab Disord* **8** 21-29.

Maggi, CA 1995 The mammalian tachykinin receptors. *Gen Pharmacol* **26** 911-944.
Maggio, JE 1988 Tachykinins. *Annu Rev Neurosci* **11** 13-28.
Micevych, PE, DW Matt, and VL Go 1988 Concentrations of cholecystokinin, substance P, and bombesin in discrete regions of male and female rat brain: sex differences and estrogen effects. *Exp Neurol* **100** 416-425.
Mittelman-Smith, MA, H Williams, SJ Krajewski-Hall, J Lai, P Ciofi, NT McMullen, and NE Rance 2012 Arcuate kisspeptin/neurokinin B/dynorphin (KNDy) neurons mediate the estrogen suppression of gonadotropin secretion and body weight. *Endocrinology* **153** 2800-2812.
Moenter, SM, AR DeFazio, GR Pitts, and CS Nunemaker 2003 Mechanisms underlying episodic gonadotropin-releasing hormone secretion. *Front Neuroendocrinol* **24** 79-93.
Muntz, JA, JD Rose, and RC Shults 1980 Disruption of lordosis by dorsal midbrain lesions in the golden hamster. *Brain Res Bull* **5** 359-364.
Narayanaswamy, S, JK Prague, CN Jayasena, DA Papadopoulou, M Mizamtsidi, AJ Shah, P Bassett, AN Comninos, A Abbara, SR Bloom, JD Veldhuis, and WS Dhillo 2016 Investigating the KNDy Hypothesis in Humans by Coadministration of Kisspeptin, Neurokinin B, and Naltrexone in Men. *J Clin Endocrinol Metab* **101** 3429-3436.
Navarro, VM 2012 New insights into the control of pulsatile GnRH release: the role of Kiss1/neurokinin B neurons. *Front Endocrinol (Lausanne)* **3** 48.
Navarro, VM 2013 Interactions between kisspeptins and neurokinin B. *Adv Exp Med Biol* **784** 325-347.
Navarro, VM, MA Bosch, S Leon, S Simavli, C True, L Pinilla, RS Carroll, SB Seminara, M Tena-Sempere, OK Ronnekleiv, and UB Kaiser 2015 The integrated hypothalamic tachykinin-kisspeptin system as a central coordinator for reproduction. *Endocrinology* **156** 627-637.
Navarro, VM, JM Castellano, R Fernandez-Fernandez, ML Barreiro, J Roa, JE Sanchez-Criado, E Aguilar, C Dieguez, L Pinilla, and M Tena-Sempere 2004 Developmental and hormonally regulated messenger ribonucleic acid expression of KiSS-1 and its putative receptor, GPR54, in rat hypothalamus and potent luteinizing hormone-releasing activity of KiSS-1 peptide. *Endocrinology* **145** 4565-4574.
Navarro, VM, JM Castellano, SM McConkey, R Pineda, F Ruiz-Pino, L Pinilla, DK Clifton, M Tena-Sempere, and RA Steiner 2011a Interactions between kisspeptin and neurokinin B in the control of GnRH secretion in the female rat. *Am J Physiol Endocrinol Metab* **300** E202-210.
Navarro, VM, ML Gottsch, C Chavkin, H Okamura, DK Clifton, and RA Steiner 2009 Regulation of gonadotropin-releasing hormone secretion by kisspeptin/dynorphin/neurokinin B neurons in the arcuate nucleus of the mouse. *J Neurosci* **29** 11859-11866.
Navarro, VM, ML Gottsch, M Wu, D Garcia-Galiano, SJ Hobbs, MA Bosch, L Pinilla, DK Clifton, A Dearth, OK Ronnekleiv, RE Braun, RD Palmiter, M Tena-Sempere, M Alreja, and RA Steiner 2011b Regulation of NKB pathways and their roles in the control of Kiss1 neurons in the arcuate nucleus of the male mouse. *Endocrinology* **152** 4265-4275.
Navarro, VM, F Ruiz-Pino, MA Sanchez-Garrido, D Garcia-Galiano, SJ Hobbs, M Manfredi-Lozano, S Leon, S Sangiao-Alvarellos, JM Castellano, DK Clifton, L Pinilla, RA Steiner, and M Tena-Sempere 2012 Role of neurokinin B in the control of female puberty and its modulation by metabolic status. *J Neurosci* **32** 2388-2397.
Noritake, K, T Matsuoka, T Ohsawa, K Shimomura, A Sanbuissho, Y Uenoyama, K Maeda, and H Tsukamura 2011 Involvement of neurokinin receptors in the control of pulsatile luteinizing hormone secretion in rats. *J Reprod Dev* **57** 409-415.

Oakley, AE, DK Clifton, and RA Steiner 2009 Kisspeptin signaling in the brain. *Endocr Rev* **30** 713-743.

Ohkura, S, K Takase, S Matsuyama, K Mogi, T Ichimaru, Y Wakabayashi, Y Uenoyama, Y Mori, RA Steiner, H Tsukamura, KI Maeda, and H Okamura 2009 Gonadotrophin-releasing hormone pulse generator activity in the hypothalamus of the goat. *J Neuroendocrinol* **21** 813-821.

Ojeda, SR, and A Lomniczi 2014 Puberty in 2013: Unravelling the mystery of puberty. *Nat Rev Endocrinol* **10** 67-69.

Ojeda, SR, A Lomniczi, U Sandau, and V Matagne 2010 New concepts on the control of the onset of puberty. *Endocr Dev* **17** 44-51.

Olster, DH, and JD Blaustein 1992 Estradiol pulses induce progestin receptors selectively in substance P-immunoreactive neurons in the ventrolateral hypothalamus of female guinea pigs. *J Neurobiol* **23** 293-301.

Otsuka, M, and K Yoshioka 1993 Neurotransmitter functions of mammalian tachykinins. *Physiol Rev* **73** 229-308.

Page, NM 2005 New challenges in the study of the mammalian tachykinins. *Peptides* **26** 1356-1368.

Patacchini, R, and CA Maggi 2001 Peripheral tachykinin receptors as targets for new drugs. *Eur J Pharmacol* **429** 13-21.

Pfaff, DW, and Y Sakuma 1979 Deficit in the lordosis reflex of female rats caused by lesions in the ventromedial nucleus of the hypothalamus. *J Physiol* **288** 203-210.

Pinilla, L, E Aguilar, C Dieguez, RP Millar, and M Tena-Sempere 2012 Kisspeptins and reproduction: physiological roles and regulatory mechanisms. *Physiol Rev* **92** 1235-1316.

Plant, TM, LC Krey, J Moossy, JT McCormack, DL Hess, and E Knobil 1978 The arcuate nucleus and the control of gonadotropin and prolactin secretion in the female rhesus monkey (Macaca mulatta). *Endocrinology* **102** 52-62.

Porter, KL, SM Hileman, SL Hardy, CC Nestor, MN Lehman, and RL Goodman 2014 Neurokinin-3 receptor activation in the retrochiasmatic area is essential for the full pre-ovulatory luteinising hormone surge in ewes. *J Neuroendocrinol* **26** 776-784.

Radovick, S, JE Levine, and A Wolfe 2012 Estrogenic regulation of the GnRH neuron. *Front Endocrinol (Lausanne)* **3** 52.

Ramaswamy, S, SB Seminara, B Ali, P Ciofi, NA Amin, and TM Plant 2010 Neurokinin B stimulates GnRH release in the male monkey (Macaca mulatta) and is colocalized with kisspeptin in the arcuate nucleus. *Endocrinology* **151** 4494-4503.

Ramaswamy, S, SB Seminara, and TM Plant 2011 Evidence from the agonadal juvenile male rhesus monkey (Macaca mulatta) for the view that the action of neurokinin B to trigger gonadotropin-releasing hormone release is upstream from the kisspeptin receptor. *Neuroendocrinology* **94** 237-245.

Rance, NE, and TR Bruce 1994 Neurokinin B gene expression is increased in the arcuate nucleus of ovariectomized rats. *Neuroendocrinology* **60** 337-345.

Rance, NE, and WS Young, 3rd 1991 Hypertrophy and increased gene expression of neurons containing neurokinin-B and substance-P messenger ribonucleic acids in the hypothalami of postmenopausal women. *Endocrinology* **128** 2239-2247.

Ricciardi, KH, and JD Blaustein 1994 Projections from ventrolateral hypothalamic neurons containing progestin receptor- and substance P-immunoreactivity to specific forebrain and midbrain areas in female guinea pigs. *J Neuroendocrinol* **6** 135-144.

Roa, J 2013 Role of GnRH Neurons and Their Neuronal Afferents as Key Integrators between Food Intake Regulatory Signals and the Control of Reproduction. *Int J Endocrinol* **2013** 518046.

Ronnekleiv, OK, MJ Kelly, and RL Eskay 1984 Distribution of immunoreactive substance P neurons in the hypothalamus and pituitary of the rhesus monkey. *J Comp Neurol* **224** 51-59.

Rubin, BS, and RJ Barfield 1983 Induction of estrous behavior in ovariectomized rats by sequential replacement of estrogen and progesterone to the ventromedial hypothalamus. *Neuroendocrinology* **37** 218-224.

Ruiz-Pino, F, D Garcia-Galiano, M Manfredi-Lozano, S Leon, MA Sanchez-Garrido, J Roa, L Pinilla, VM Navarro, and M Tena-Sempere 2015 Effects and interactions of tachykinins and dynorphin on FSH and LH secretion in developing and adult rats. *Endocrinology* **156** 576-588.

Ruiz-Pino, F, VM Navarro, AH Bentsen, D Garcia-Galiano, MA Sanchez-Garrido, P Ciofi, RA Steiner, JD Mikkelsen, L Pinilla, and M Tena-Sempere 2012 Neurokinin B and the control of the gonadotropic axis in the rat: developmental changes, sexual dimorphism, and regulation by gonadal steroids. *Endocrinology* **153** 4818-4829.

Ruka, KA, LL Burger, and SM Moenter 2016 Both Estrogen and Androgen Modify the Response to Activation of Neurokinin-3 and kappa-Opioid Receptors in Arcuate Kisspeptin Neurons From Male Mice. *Endocrinology* **157** 752-763.

Saffroy, M, Y Torrens, J Glowinski, and JC Beaujouan 2003 Autoradiographic distribution of tachykinin NK2 binding sites in the rat brain: comparison with NK1 and NK3 binding sites. *Neuroscience* **116** 761-773.

Sahu, A, and SP Kalra 1992 Effects of tachykinins on luteinizing hormone release in female rats: potent inhibitory action of neuropeptide K. *Endocrinology* **130** 1571-1577.

Sandoval-Guzman, T, and NE Rance 2004 Central injection of senktide, an NK3 receptor agonist, or neuropeptide Y inhibits LH secretion and induces different patterns of Fos expression in the rat hypothalamus. *Brain Res* **1026** 307-312.

Seabrook, GR, BJ Bowery, and RG Hill 1995 Pharmacology of tachykinin receptors on neurones in the ventral tegmental area of rat brain slices. *Eur J Pharmacol* **273** 113-119.

Seminara, SB, S Messager, EE Chatzidaki, RR Thresher, JS Acierno, Jr., JK Shagoury, Y Bo-Abbas, W Kuohung, KM Schwinof, AG Hendrick, D Zahn, J Dixon, UB Kaiser, SA Slaugenhaupt, JF Gusella, S O'Rahilly, MB Carlton, WF Crowley, Jr., SA Aparicio, and WH Colledge 2003 The GPR54 gene as a regulator of puberty. *N Engl J Med* **349** 1614-1627.

Shamgochian, MD, and SE Leeman 1992 Substance P stimulates luteinizing hormone secretion from anterior pituitary cells in culture. *Endocrinology* **131** 871-875.

Simavli, S, IR Thompson, CA Maguire, JC Gill, RS Carroll, A Wolfe, UB Kaiser, and VM Navarro 2015 Substance p regulates puberty onset and fertility in the female mouse. *Endocrinology* **156** 2313-2322.

Smith, JT, HM Dungan, EA Stoll, ML Gottsch, RE Braun, SM Eacker, DK Clifton, and RA Steiner 2005 Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse. *Endocrinology* **146** 2976-2984.

Steiner, RA, and VM Navarro 2012 Tacking toward reconciliation on Tacr3/TACR3 mutations. *Endocrinology* **153** 1578-1581.

Stephens, SB, N Chahal, N Munaganuru, RA Parra, and AS Kauffman 2016 Estrogen stimulation of Kiss1 expression in the medial amygdala involves estrogen receptor alpha but not estrogen receptor beta. *Endocrinology* en20161431.

Teles, MG, SD Bianco, VN Brito, EB Trarbach, W Kuohung, S Xu, SB Seminara, BB Mendonca, UB Kaiser, and AC Latronico 2008 A GPR54-activating mutation in a patient with central precocious puberty. *N Engl J Med* **358** 709-715.

Topaloglu, AK, F Reimann, M Guclu, AS Yalin, LD Kotan, KM Porter, A Serin, NO Mungan, JR Cook, MN Ozbek, S Imamoglu, NS Akalin, B Yuksel, S O'Rahilly, and RK Semple 2009 TAC3 and TACR3 mutations in familial hypogonadotropic hypogonadism

reveal a key role for Neurokinin B in the central control of reproduction. *Nat Genet* **41** 354-358.

Topaloglu, AK, JA Tello, LD Kotan, MN Ozbek, MB Yilmaz, S Erdogan, F Gurbuz, F Temiz, RP Millar, and B Yuksel 2012 Inactivating KISS1 mutation and hypogonadotropic hypogonadism. *N Engl J Med* **366** 629-635.

Traczyk, WZ, KY Pau, AH Kaynard, and HG Spies 1992 Modulatory role of substance P on gonadotropin and prolactin secretion in the rabbit. *J Physiol Pharmacol* **43** 279-297.

True, C, S Nasrin Alam, K Cox, YM Chan, and S Seminara 2015 Neurokinin B is critical for normal timing of sexual maturation but dispensable for adult reproductive function in female mice. *Endocrinology* en20141862.

Tsuruo, Y, S Hisano, J Nakanishi, S Katoh, and S Daikoku 1987 Immunohistochemical studies on the roles of substance P in the rat hypothalamus: possible implication in the hypothalamic-hypophysial-gonadal axis. *Neuroendocrinology* **45** 389-401.

Tsuruo, Y, H Kawano, S Hisano, Y Kagotani, S Daikoku, T Zhang, and N Yanaihara 1991 Substance P-containing neurons innervating LHRH-containing neurons in the septo-preoptic area of rats. *Neuroendocrinology* **53** 236-245.

Vink, R, and C van den Heuvel 2010 Substance P antagonists as a therapeutic approach to improving outcome following traumatic brain injury. *Neurotherapeutics* **7** 74-80.

Wakabayashi, Y, T Nakada, K Murata, S Ohkura, K Mogi, VM Navarro, DK Clifton, Y Mori, H Tsukamura, K Maeda, RA Steiner, and H Okamura 2010 Neurokinin B and dynorphin A in kisspeptin neurons of the arcuate nucleus participate in generation of periodic oscillation of neural activity driving pulsatile gonadotropin-releasing hormone secretion in the goat. *J Neurosci* **30** 3124-3132.

Weems, PW, CF Witty, M Amstalden, LM Coolen, RL Goodman, and MN Lehman 2016 kappa-Opioid Receptor Is Colocalized in GnRH and KNDy Cells in the Female Ovine and Rat Brain. *Endocrinology* **157** 2367-2379.

Yamamura, T, Y Wakabayashi, S Ohkura, VM Navarro, and H Okamura 2015 Effects of intravenous administration of neurokinin receptor subtype-selective agonists on gonadotropin-releasing hormone pulse generator activity and luteinizing hormone secretion in goats. *J Reprod Dev* **61** 20-29.

Yamano, M, S Inagaki, S Kito, and M Tohyama 1986 A substance P-containing pathway from the hypothalamic ventromedial nucleus to the medial preoptic area of the rat: an immunohistochemical analysis. *Neuroscience* **18** 395-402.

Yamanouchi, K, Y Nakano, and Y Arai 1990 Roles of the pontine dorsomedial tegmentum and midbrain central gray in regulating female rat sexual behaviors: effects of p-chlorophenylalanine. *Brain Res Bull* **25** 381-385.

Yang, JJ, CS Caligioni, YM Chan, and SB Seminara 2012 Uncovering novel reproductive defects in neurokinin B receptor null mice: closing the gap between mice and men. *Endocrinology* **153** 1498-1508.

Young, J, J Bouligand, B Francou, ML Raffin-Sanson, S Gaillez, M Jeanpierre, M Grynberg, P Kamenicky, P Chanson, S Brailly-Tabard, and A Guiochon-Mantel 2010 TAC3 and TACR3 defects cause hypothalamic congenital hypogonadotropic hypogonadism in humans. *J Clin Endocrinol Metab* **95** 2287-2295.

Zacest, AC, R Vink, J Manavis, GT Sarvestani, and PC Blumbergs 2010 Substance P immunoreactivity increases following human traumatic brain injury. *Acta Neurochir Suppl* **106** 211-216.