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

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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

40 autonomous activity (spontaneous bursts), these do not seem to correlate with GnRH/LH pulses

41 in vivo [reviewed in (Navarro 2012)]. Furthermore, GnRH neurons lack the ability to sense most

42 factors that influence reproductive function, such as endogenous signals [e.g. sex steroid

43 hormones; (Hrabovszky and Liposits 2013, Radovick, et al. 2012, Roa 2013)] as well as

44 environmental cues [e.g. stressors; (Dobson, et al. 2003)]. Thus, a large body of research is now

45 focusing on the discovery of higher hierarchy circuits and their efficacy in stimulating GnRH

46 secretion in to the hypophyseal portal vessels, thereby enabling gonadotropin [luteinizing

47 hormone (LH) and follicle stimulating hormone (FSH)] secretion from the anterior pituitary in to

48 the peripheral circulation. From then on, LH and FSH reach the gonads to stimulate

49 gametogenesis and sex steroid production. In turn, sex steroids exert positive and negative

50 feedback effects on pituitary and hypothalamic target cells (Herbison 1998), completing the H-P-

51 G axis. In this respect, over the past 10 years, several upstream neurophenotypes have been

52 implicated in stimulatory and/or inhibitory regulation of GnRH secretion.

53 The path was initially paved with the discovery that loss-of-function mutations in several

54 neuroendocrine genes, including *KISS1* and its receptor, *KISS1R* (Table 1), have been described

55 to cause hypogonadotropic hypogonadism in humans (Chan, et al. 2011, de Roux, et al. 2003,

56 Seminara, et al. 2003, Topaloglu, et al. 2012) due to a central deficit that leads to absent GnRH/LH

57 pulses, highlighting the importance of these neural cues in GnRH release. Further anatomical and

58 functional studies provided unequivocal evidence that kisspeptins, encoded by the *Kiss1* gene

59 (Table 1), are the most potent secretagogues of GnRH in all mammals studied to date (Oakley,

60 et al. 2009). A number of studies by our lab and others suggest that Kiss1 neurons – which contact

61 GnRH neurons directly - receive profuse central and peripheral regulatory inputs that modulate

62 kisspeptin secretion for the initiation of puberty and the maintenance of fertility in adulthood

63 (Pinilla, et al. 2012, Seminara, et al. 2003). Importantly, Kiss1 neurons also play a critical role in

64 conveying information about the sex steroid milieu to GnRH neurons (Gill, et al. 2010, Navarro,

65   et al. 2004). However, kisspeptin action on GnRH neurons is necessary but not sufficient for the

66   proper activation of GnRH neurons (Leon, et al. 2016).

67   The development of newer, more potent and less expensive tools to screen genome sequences

68   of affected patients is revealing a growing number of factors that appear critical for the timing of

69   puberty onset and maintenance of fertility by regulating kisspeptin and/or GnRH/LH release.

70   Within this constellation of neuroendocrine systems, is the one comprised by the tachykinin

71   neurokinin B (NKB) and its receptor (NK3R), encoded by *TAC3* and *TACR3* in humans,

72   respectively (Table 1). This system has received substantial attention since the identification in

73   2009 of inactivating mutations in these genes are also associated with hypogonadotropic

74   hypogonadism and lack of puberty onset (Topaloglu, et al. 2009, Topaloglu, et al. 2012, Yang, et

75   al. 2012, Young, et al. 2010), resembling the phenotype of *KISS1/KISS1R* null patients. Moreover,

76   the systemic administration of an NK3R antagonist (ESN364) in OVX ewes, castrated or cycling

77   nonhuman primates as well as healthy men and women (Fraser, et al. 2015, Fraser, et al. 2016)

78   show a partial inhibition of the reproductive axis. Indeed, numerous follow-up animal studies,

79   confirmed that NKB is a critical stimulatory input to the GnRH network, in various species

80   (Goodman, et al. 2014, Navarro 2013) although, interestingly, this stimulatory effect is not

81   observed in healthy men (Narayanaswamy, et al. 2016), probably due to their circulating sex

82   steroid levels as discussed below. However, unlike kisspeptin deficiency, the phenotype of

83   patients lacking NKB signaling is less severe since reversal cases have been documented, in

84   which some patients recovered reproductive function and fertility after delayed puberty (Gianetti,

85   et al. 2010). A similar subfertile phenotype has been observed in genetically modified mouse

86   models, where *Tac2* and *Tacr3* (encoding NKB and NK3R, respectively, in rodents, Table 1) had

87   been deleted from the genome (Steiner and Navarro 2012, True, et al. 2015, Yang, et al. 2012).

88   Therefore, it appears that the reversal phenotype in reproductive viability observed in human

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89   individuals with *TAC3/TACR3* or rodents with *Tac2/Tacr3* mutations may be due to compensation

90   by other neuronal systems.

91   Interestingly, NKB is a member of the broader tachykinin family, which has the common C-

92   terminal sequence of Phe-X-Gly-Leu-Met-NH$_2$ (Maggio 1988). This family also includes

93   substance P (SP), neurokinin A (NKA), neuropeptide K (NPK), and neuropeptide γ (NPγ) (Otsuka

94   and Yoshioka 1993, Page 2005). The vast majority of research has focused on SP, NKA and NKB

95   which bind preferentially to the NK1R, NK2R and NK3R G-protein coupled receptors, respectively

96   (Maggi 1995, Patacchini and Maggi 2001, Saffroy, et al. 2003).

97   Early studies documented a robust stimulatory action of LH release by SP in rats, rabbits and

98   humans (Arisawa, et al. 1990, Coiro, et al. 1992, Kalra, et al. 1992, Sahu and Kalra 1992, Traczyk,

99   et al. 1992) and recent electrophysiological studies have described potent depolarizing effects of

100 SP and NKA on ARC Kiss1 neurons in the mouse (de Croft, et al. 2013) indicating that LH

101 stimulation by these tachykinins involves, at least in part, a kisspeptin dependent mechanism. Of

102 note, this study showed that, *in vitro*, the activation of kisspeptin neurons by NKB was completely

103 diminished only when all three neurokinin receptor (NKR) subtype-selective antagonists were

104 concomitantly applied in the *in vitro* bath (de Croft, et al. 2013). This is in line with studies carried

105 out *in vivo* indicating that blockade of all 3 tachykinin receptors (but not each one of them

106 individually) prevented the compensatory rise of LH after gonadectomy (GDX) in rats (Noritake,

107 et al. 2011). Therefore, considerable cross reactivity exists between these receptor/ligand

108 systems and each one of these neuropeptides is capable of eliciting responses from all three

109 neurokinin receptors (Beaujouan, et al. 2000, Cascieri, et al. 1992, Gether, et al. 1993). In these

110 studies, the affinities or EC50 values of each tachykinin for NK1R, NK2R, and NK3R, respectively,

111 were reported as follows: SP_2nM, 2200nM, and 18000nM; NKA _ 16nM, 3nM, and 1300nM; and

112 NKB_70nM, 25nM, and 4nM (Seabrook, et al. 1995). These data suggest a likely interaction of

113 NKA with NK1R as well as NK2R, and of NKB with all 3 receptors, at relatively low concentrations.

114 Furthermore, it has been demonstrated in rats, that pulsatile LH secretion was suppressed by

115 central administration of CS-003, an antagonist for all three NKRs, whereas administration of

116 each NKR subtype-selective antagonist alone, had no effect (Noritake, et al. 2011). In this respect,

117 several pieces of evidence will be discussed below that provide unequivocal evidence that other

118 members of the tachykinin family, namely SP and NKA, all encoded by the *TAC1 or Tac1* gene

119 (Table 1), in humans and rodents respectively (Lasaga and Debeljuk 2011) are an important

120 component of the integrated neuronal hypothalamic system that controls GnRH/LH secretion in

121 mammals.

The current model for the GnRH pulse generator.

123 Kiss1 neurons are located primarily in two discrete hypothalamic nuclei: the arcuate nucleus

124 (ARC) and the anteroventral periventricular nucleus (AVPV/PeN) in rodents (Clarkson, et al.

125 2009) or the preoptic area in ruminants (Lehman, et al. 2010), monkeys (Luque, et al. 2011) and

126 humans (Hrabovszky 2014). Compelling evidence suggests that Kiss1 neurons in the ARC

127 mediate the negative feedback of sex steroids and *Kiss1* expression is inhibited by estradiol (E_2)

128 and testosterone (T). By contrast, *Kiss1* expression in the AVPV/PeN—almost exclusive to the

129 female brain— is upregulated by E_2 and mediate the positive feedback that leads to the female-

130 specific preovulatory GnRH/LH surge (Maeda, et al. 2007, Navarro, et al. 2004, Smith, et al.

131 2005). Substantial *in vivo* and *in vitro* evidence points to the importance of a population of neurons

132 located in the ARC of the hypothalamus in playing the role of the GnRH pulse generator. The

133 notion originated from studies carried out in the ovariectomized (OVX) rhesus monkey, in which

134 LH secretion was abolished by selective lesioning of the ARC (Plant, et al. 1978), and was further

135 reinforced by findings that multiunit electrical activity (MUA) in the vicinity of ARC kiss1 neurons

136 was tightly coupled LH pulses (Kawakami, et al. 1982, Ohkura, et al. 2009). In this context, Kiss1

137 neurons in the ARC coexpress dynorphin (inhibitory) and NKB (stimulatory) referred to as KNDy

138 neurons (Cheng, et al. 2010, Goodman, et al. 2013, Navarro 2012), which have been proposed

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139   to act in a coordinated, reciprocal fashion to shape the pulsatile release of kisspeptin in the median

140   eminence, which in turn induces corresponding intermittent GnRH discharges at this site (Keen,

141   et al. 2008). This has since been demonstrated in a variety of mammals including mice (Navarro,

142   et al. 2009), rats (Navarro, et al. 2011a), sheep (Goodman, et al. 2013), goats (Wakabayashi, et

143   al. 2010) and monkeys (Ramaswamy, et al. 2010). In this model, NKB would stimulate kisspeptin

144   release and dynorphin would then inhibit this release through autosynaptic loops, thus shaping a

145   kisspeptin/GnRH/LH pulse (Keen, et al. 2008). This is supported by the anatomical findings that

146   virtually all KNDy neurons express NK3R (Amstalden, et al. 2010, Navarro, et al. 2009, Navarro,

147   et al. 2011b) and >90% express kappa-opioid receptor [KOR; (Weems, et al. 2016)]. Furthermore,

148   KNDy cells are interconnected with NKB fibers within the ARC forming a tightly regulated network

149   (Krajewski, et al. 2010, Lehman, et al. 2010, Rance and Bruce 1994). Indeed, a growing number

150   of studies in multiple species from our lab and others support the ability of NKB -or the NKB

151   receptor (NK3R) agonist senktide- to increase LH pulses (Goodman, et al. 2014, Grachev, et al.

152   2012, Navarro 2013). This places the KNDy neurons as ideal candidates for the role of the GnRH

153   pulse generator. However, more recently, several studies have provided evidence that other

154   tachykinins, i.e., SP and NKA, merit further investigation as additional fundamental components

155   of the current, KNDy-dominated, GnRH pulse generator model. Although no human mutations in

156   the genes encoding SP and NKA (*TAC1*) or their receptors (*TACR1* and *TACR2*, respectively;

157   Table 1) have been correlated with reproductive disorders yet, both SP and NKA have been

158   reported to stimulate the gonadotropic axis in several species (Arisawa, et al. 1990, de Croft, et

159   al. 2013, Kalra, et al. 1992, Navarro, et al. 2015, Noritake, et al. 2011, Sahu and Kalra 1992)

160   including men (Coiro, et al. 1992). It is therefore plausible to speculate that these tachykinins are

161   involved in the central regulation of GnRH release and may be additional elements to the GnRH

162   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

187 and Young 1991, Ronnekleiv, et al. 1984, Tsuruo, et al. 1991, Yamano, et al. 1986). Studies

188 employing immunohistochemical detection of SP also report a plethora of fibers that innervate the

189 entire length of the ARC and the median eminence (ME) (Hrabovszky, et al. 2013, Kalil, et al.

190 2015) which appear to surround the capillaries of the hypophyseal portal system indicating that

191 SP may have the ability to act directly on the anterior pituitary (Kalil, et al. 2015).

192 Interestingly, even though the *Tac2* (gene encoding NKB; Table 1) is known to be coexpressed

193 within Kiss1 in the ARC of various species, including humans (Goodman, et al. 2007, Hrabovszky

194 2014, Navarro, et al. 2009) the *Tac1*-positive neurons did not colocalize with *Kiss1*-positive

195 neurons in the mouse [(Navarro, et al. 2015); Figure.1). This is in agreement with equivalent

196 investigations in the monkey (Kalil, et al. 2015) and rat (Rance and Bruce 1994) but contradict

197 findings in the human that report approximately 65% of SP neurons in the ARC coexpress

198 kisspeptin [conversely, 30% of Kiss1 neurons contain SP; (Hrabovszky, et al. 2013)]. The reason

199 for this divergence is not known, however, it supports the notion for the existence of potential

200 differences in the function of the tachykinin systems across species (Hrabovszky, et al. 2013,

201 Kalil, et al. 2015, Navarro, et al. 2015). Nonetheless, the population of *Tac1* neurons in the ARC

202 of the mouse (Navarro, et al. 2015) and SP immunoreactive neurons and fibers in the monkey

203 (Kalil, et al. 2015) appeared to be in close contact with Kiss1 neurons and fibers [and GnRH fibers

204 as shown in postmenopausal women (Hrabovszky, et al. 2013)] in the ARC, presumably

205 facilitating the interaction between all three neuronal populations. Immunohistochemical analysis

206 of NKA fiber colocalization with kisspeptin or GnRH afferents merits future investigation. Of note,

207 *Tac1* mRNA was not detected in the AVPV/PeN of mice (Figure. 1), the region in which the second

208 population of Kiss1 neurons reside (Oakley, et al. 2009), however, data from other species is non-

209 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

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235 GnRH surge is likely, but remains unexplored. The action of NKA, on the other hand, remains

236 largely unresolved, because *Tacr2* has been identified in neither Kiss1 nor GnRH neurons, thus,

237 suggesting the presence of unidentified intermediate upstream neurons [(Navarro, et al. 2015);

238 Figure.1].

239 ## Sex steroid regulation of SP and NKA.

240 All known cotransmitters present in ARC Kiss1 neurons (Kiss1, NKB, and dynorphin) are inhibited

241 by sex steroids as part of their hypothesized role in the negative feedback upon GnRH release

242 (Gottsch, et al. 2009, Navarro, et al. 2009). This also appears to be true for SP and NKA, as *Tac1-*

243 expressing neurons in the ARC and VMN of mice were downregulated by OVX and E_2 treatment

244 (Micevych, et al. 1988, Navarro, et al. 2015) and immunopositive SP protein in the ARC increased

245 after gonadectomy (GND) in the male monkey (Kalil, et al. 2015). Furthermore, this effect

246 appeared to be specific for these areas of the brain (Navarro, et al. 2015) and was not evident

247 elsewhere. Similarly, SP mRNA increased in the hypothalamus of post- compared to pre-

248 menopausal women (Rance and Young 1991) and the content of SP in the ARC has been shown

249 to increase after OVX in the rat (Tsuruo, et al. 1987). The results of all these studies suggest that

250 downregulation of SP and NKA in hypothalamic neurons may mediate, at least in part, the

251 negative feedback action of gonadal steroids on gonadotropin secretion. Indeed, earlier studies

252 have demonstrated that a substantial population of SP immunoreactive cells located in the

253 mediobasal hypothalamus of the rat are estrogen receptive (26.1% in the Arc and 42.9% in the

254 VMN) (Akesson and Micevych 1988). Interestingly, immunohistochemical studies on human

255 hypothalami have revealed that postmenopausal women have higher numbers of SP neurons

256 and darker labeling than in age-matched men (Hrabovszky, et al. 2013). However, if this

257 constitutes a sex difference in the expression of SP or it is a mere reflection of different levels of

258 sex steroids, remains to be elucidated. In this context, an earlier report documents greater SP

259 immunoreactivity in the medial amygdala of male compared to female rats (Micevych, et al. 1988),

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260 an area which is also known for a greater Kiss1 population of cells in males versus females

261 (Stephens, et al. 2016). However, the interaction between these two systems (SP and Kiss1 in

262 the medial amygdala) has not yet been explored. Nonetheless, sex differences in the expression

263 of SP or NKA require further characterization across multiple species.

Regulation of LH release by tachykinins: sex steroid dependent

action.

Neurokinin B

267 Most studies carried out to date looking into the effect of tachykinins on reproductive function

268 have focused on the role of NKB, and less so on other members of the tachykinin family.

269 Therefore, it is useful to compare findings from SP and NKA studies with those already carried

270 out for NKB, as a synergistic action is highly probable. One thing that can be said about the

271 stimulatory effect of NKB on LH release, is that it is less robust than that of kisspeptin, and

272 inhibitory actions or null effects on LH secretion have also been documented, depending on the

273 species and the sex steroid levels (Navarro, et al. 2011a, Ruiz-Pino, et al. 2012, Sandoval-

274 Guzman and Rance 2004). For instance, NKB induced significantly stimulatory LH responses in

275 adult female rats and mice under physiological levels of sex steroids, whereas only adult intact

276 male mice (but not rats) displayed LH responses to the same challenge (Navarro, et al. 2011b,

277 Ruiz-Pino, et al. 2012). By contrast, predominant inhibitory effects of the selective NK3R agonist,

278 senktide, have been reported in rodents with null or low sex steroids levels (Grachev, et al. 2012,

279 Navarro, et al. 2015, Navarro, et al. 2011b), even though kisspeptins are known to stimulate

280 gonadotropin secretion irrespective of the sex steroid milieu (Oakley, et al. 2009). From a

281 mechanistic point of view, the inhibitory action of NKB on LH release appears to be opioid

282 mediated, as has been shown by lack of LH inhibition by senktide in the presence of KOR agonist

283 in rats (Kinsey-Jones, et al. 2012). In accordance, extracellular recordings from KNDy neurons

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284 demonstrated that gonadal feedback (by both estrogen and dihydrotestosterone) attenuates the

285 stimulatory effects of senktide on the firing rate of KNDy neurons while increasing the inhibitory

286 effects of dynorphin by modulating the activation of NK3R and KOR (Ruka, et al. 2016).

287 Interestingly, in the sheep, NKB/NK3R signaling may also be important in the generation of the

288 preovulatory GnRH/LH surge. For example, intracerebroventricular (i.c.v) microinjections of

289 senktide, in this species, results in a surge-like elevation of LH during the follicular but not the

290 luteal phase of the ovine estrous cycle (Billings, et al. 2010, Porter, et al. 2014), replicating a

291 potential dual effect of NKB, dependent on sex steroid levels, as observed in rodents (Navarro,

292 et al. 2011a). These observations illustrate the complexity of the effects of NKB on the

293 gonadotropic axis.

294

Substance P

296 To date, SP has largely been associated with processes unrelated to reproductive function, such

297 as pain perception and inflammatory activity in the brain (De Felipe, et al. 1998) as well as with

298 psychiatric disorders (Ebner and Singewald 2006). Even though SP was originally identified in

299 the 1930's (Lasaga and Debeljuk 2011) it is only now beginning to come in to the spotlight as a

300 regulator of the reproductive axis. Few earlier studies aimed to investigate the effects of SP on

301 the gonadotropic axis and report variable results (Table 2). These include peripheral (i.v.)

302 administration of SP for 1 hour in normal men, which induced a robust discharge of LH (Coiro, et

303 al. 1992) and in OVX rats i.c.v specific antiserum against SP (anti-SP) decreased plasma LH,

304 whereas synthetic SP injected i.c.v. or i.v. into OVX+E_2 rats, stimulated LH release, via both routes

305 of administration (Arisawa, et al. 1990). Other studies conducted by Kalra et al., in the 90's (Kalra,

306 et al. 1992) (Sahu and Kalra 1992) report null or inhibitory effects in intact and GND males,

307 respectively, hinting at potential sex differences in the response to SP (Table 2). Further studies

308 conducted on intact and OVX rabbits report that although the stimulatory effect of SP on LH is

309 sex steroid-independent, in the absence of ovarian steroids, SP is stimulatory only during the

310 rising phase of an LH pulse (Traczyk, et al. 1992). Interest in SP has recently rekindled and

311 studies in mice are pointing towards a clear stimulatory action on LH secretion, which appears to

312 be independent of the sex steroid milieu Table 2; (Navarro, et al. 2015)]. In this study, the

313 activation of NK1R with the i.c.v. administration of an NK1R specific agonist (GR73632) induced

314 LH release in intact males, diestrous or OVX females and a 20-fold increase in OVX+E_2 females

315 (Navarro, et al. 2015). However, in rats that received the same agonist i.c.v., with the same dose,

316 no alteration in LH levels was observed in either sex with intact gonads (Ruiz-Pino, et al. 2015)

317 indicating a potential species difference. This notion is also supported by pharmacological data

318 from ovary-intact anestrous ewes and OVX and OVX+E2 goats demonstrating that much higher

319 doses of SP are needed to stimulate LH secretion compared to those needed with senktide

320 (Goodman 2015, Yamamura, et al. 2015).

321 In addition, a small body of literature has focused on the role of SP on the LH surge as well as

322 sexual behavior. Intriguingly, a number of reports by Kerdelhué et al., in humans, monkeys and

323 rats have shown variable results. Initially, a study carried out in cycling rats, investigated the

324 effects of a subcutaneous injection of SP during proestrus, which led to a reduction of the LH

325 surge amplitude (Duval, et al. 1996). Furthermore, this inhibitory effect was reversed with the

326 simultaneous administration of SP and an NK1R antagonist (RP 67580) (Duval, et al. 1996).

327 However, further studies showed a divergence in results using the NK1R antagonist (RPR

328 100893) in OVX + E_2 treated *versus* intact cycling monkeys. In the first study, the NK1R antagonist

329 was administered in OVX + E_2 treated monkeys causing a 50% enhancement of the LH surge

330 (Kerdelhue, et al. 1997), supporting an inhibitory role of SP in the LH surge mechanism, similar

331 to what was observed in the rat (Duval, et al. 1996). By contrast, the same antagonist

332 administered during the ascending phase of plasma estradiol concentrations (prior to LH surge

333 onset of cycling monkeys), resulted in a reduction in both the amplitude (41%) and the duration

334 of the preovulatory LH surge (Kerdelhue, et al. 2000), providing evidence for a stimulatory role of

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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

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361 progesterone receptors in SP neurons located in this area (Olster and Blaustein 1992) and this

362 process is necessary for the induction of lordosis (Rubin and Barfield 1983). Collectively, these

363 results suggest that SP originating in the vl VMN may participate in the onset of lordosis behavior

364 (Dornan, et al. 1990)**,** however further detailed components of the anatomy and physiology of this

365 neurocircuitry is missing.

366 ## Neurokinin A

367 By contrast, much less information is available on the other members of the tachykinin family such

368 as NKA or its two elongated peptides, NPK and NPγ. NKA is also encoded by the *Tac1* gene in

369 the rodent and preferentially binds to the NK2R (Beaujouan, et al. 2000). The NKA/NK2R

370 signaling system appears to act through different regulatory mechanisms, than those identified

371 for SP; however, it is noteworthy, that results to date have been a lot more consistent across

372 species (Table 3). Central administration of the NK2R agonist, GR64349, displayed a NKB-like

373 action in terms of LH release (the so called dual effect of senktide), showing inhibition in OVX

374 mice but clear stimulation in OVX+E_2 treated female and intact male mice (Navarro, et al. 2015).

375 Similar results have been obtained by studies conducted in male and female rats (Kalra, et al.

376 1992, Ruiz-Pino, et al. 2015, Sahu and Kalra 1992). These data indicate that NK2R and NK3R

377 may converge on a common pathway to regulate GnRH release in a sex independent but sex

378 steroid dependent manner making them ideal candidates to participate in the GnRH pulse

379 generator (Table 3). In this aspect, pharmacological studies in goats (Yamamura, et al. 2015) and

380 sheep (Goodman 2015), showed that the three NKR agonists possess the ability to induce MUA

381 volleys and an increase in LH, respectively, albeit, with a significant difference in the efficacy to

382 do so, as much higher concentrations of NK1R and NK2R agonists were required to have a similar

383 effect as NKB agonist or senktide, respectively (Goodman 2015, Yamamura, et al. 2015).

384 Therefore, a reasonable hypothesis could be that NKA (and potentially SP) participate in the pulse

385 generator by amplifying the actions of NKB. However, this requires further investigation as

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386 equivalent pulse studies are lacking in other species. Similar to what was previously suggested

387 for the inhibitory action of NKB, the inhibitory action of NKA on LH release appears to also be

388 opioid mediated, at least in the rat (Kalra, et al. 1992). It is plausible to speculate that there is a

389 sex steroid dependent differential activation of the stimulatory (NK3R) or inhibitory receptor (KOR)

390 after the administration of an NKA agonist in the presence versus absence of sex steroids,

391 however, this remains to be proven.

392

Tachykinins modulate the gonadotropic axis in a kisspeptin dependent manner.

395 It is now well recognized that the stimulating effects of NKB on GnRH secretion are mediated

396 primarily via initial kisspeptin stimulation. This has been demonstrated by studies that have shown

397 that a) desensitization of the kisspeptin receptor blocks the stimulatory effect of senktide in

398 monkeys (Ramaswamy, et al. 2011), b) senktide i.c.v administration induces c-Fos activation of

399 kisspeptin cells in the ARC of rats (Navarro, et al. 2011a), c) as mentioned above, nearly all ARC

400 kisspeptin cells contain NK3R receptors (Navarro, et al. 2009) and are excited by senktide/NKB

401 (de Croft, et al. 2013), d) the stimulatory effect of senktide, is completely absent in *Kiss1r*KO mice

402 (Garcia-Galiano, et al. 2012) and e) specific ablation of NK3R expressing neurons in the ARC of

403 the rat impairs the postcastration rise in LH secretion (Mittelman-Smith, et al. 2012). The above

404 studies clearly indicate the importance of NKB signaling on kisspeptin for GnRH stimulation.

405 However, additional regulation of GnRH release at a different level, i.e. kisspeptin-independent

406 action, cannot be excluded given the presence of NK1R and NK3R in a subset of GnRH neurons

407 (Krajewski, et al. 2005, Navarro, et al. 2015) and the reported kisspeptin-independent activation

408 of GnRH neurons by NK3R agonists *in vitro* (Gaskins, et al. 2013).

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409 In this regard, a similar mechanism of action appears to be employed by SP and NKA. Recent

410 electrophysiological studies in a kisspeptin-green fluorescent protein mouse model, have

411 described potent stimulatory actions of SP and NKA on ARC Kiss1 neurons (de Croft, et al. 2013).

412 In addition, the administration of all individual tachykinin receptor agonists to mice lacking Kiss1r

413 [*Kiss1r*KO mice] resulted in absent LH responses (Navarro, et al. 2015). This, taken together with

414 the fact that 50% of KNDy neurons contain NK1R (Navarro, et al. 2015), suggests that SP is able

415 to stimulate LH secretion by acting, at least in part, via a kisspeptin dependent mechanism

416 (Figure. 1). Intriguingly, in a recent study on female mice, NK1R agonist (GR73632) elicited a

417 greater LH response than that observed with an NK2R agonist [GR64349; (Navarro, et al. 2015)].

418 It is possible that the augmented stimulatory action of NK1R agonist on LH release is a reflection

419 of the additional action of SP on both populations of Kiss1 neurons (ARC and AVPV/PeN)

420 (Navarro, et al. 2015). In support of this hypothesis, the same exaggerated effect of NK1R agonist

421 was not observed in male mice (Navarro, et al. 2015), which also lack an AVPV kiss1 neuronal

422 population (Kauffman, et al. 2007, Smith, et al. 2005). Potential direct action on GnRH neurons

423 however, cannot be overlooked, as at least in the mouse, a subset of GnRH neurons express SP

424 (and NKB) receptors (Navarro, et al. 2015) and senktide can induce *in vitro* GnRH secretion in

425 the ME in brain slices derived from *Kiss1* knockout mice (Gaskins, et al. 2013). In this light, a very

426 important question arises, which is also true for the action of NKB, as to which pathway is

427 employed when (kisspeptin versus GnRH dependent pathways) and for what biological purpose.

428 Potentially, as the majority of studies investigating the necessity of an intact Kiss1/Kiss1r signaling

429 system in the stimulation of LH secretion by tachykinins have been carried out in the persistent

430 hypogonadal state (primarily via the blockade of kiss1r; see above), it is plausible to speculate

431 that the sex steroid milieu may be an important determining factor. Studies carried out with or

432 without the presence of sex steroids and an absent Kiss1/Kiss1r system may be useful in this

433 aspect. The action of NKA, however, is less clear, because *Tacr2* is not present in either Kiss1

434 or GnRH neurons, while showing a kisspeptin-dependent action (Navarro, et al. 2015), thus

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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

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459 periods, neurons secreting GnRH are subjected to persistent synaptic inhibition (Ojeda, et al.

460 2010). When this inhibition is removed, GnRH secretion increases, which leads to puberty.

461 However, it is recognized that a gain in numerous excitatory inputs to GnRH neurons is also

462 indispensable (Ojeda and Lomniczi 2014). In this respect, both loss-of-function and gain-of-

463 function mutations in a growing number of neurotransmitters and their receptors have been

464 described to severely impinge on the pubertal transition. As mentioned above, a number of

465 studies have documented lack or delay of pubertal maturation in humans and mice bearing loss-

466 of-function mutations in *KISS1/KISS1R* or *TAC3/TACR3* genes (de Roux, et al. 2003, Seminara,

467 et al. 2003, Topaloglu, et al. 2012, Young, et al. 2010). In contrast, gain-of function mutations in

468 KISS1R have been identified in association with central precocious puberty (Teles, et al. 2008).

469 Therefore, kisspeptins are indispensable regulatory signals of GnRH release during puberty

470 (Seminara, et al. 2003). In the same vein, the tachykinin NKB has been reported to stimulate

471 kisspeptin prepubertally (Navarro, et al. 2012) and the expression of *Tac2* increases before *Kiss1*

472 (Gill, et al. 2012), suggesting a likely role of this tachykinin in the pubertal activation of kisspeptin-

473 GnRH secretion (Topaloglu, et al. 2009, Young, et al. 2010).

474 The equivalent role of SP and NKA in the prepubertal increase of LH release and their contribution

475 to the timing of puberty onset has only recently began to draw attention. A series of functional

476 tests and genetic studies in the female mouse, have shown that SP/NK1R and NKA/NK2R

477 signaling, appears to participate in the timing of puberty. This conclusion is derived from a study

478 by (Simavli, et al. 2015) which has shown that 1) a selective NK1R agonist induces LH release in

479 prepubertal females; 2) the expression of *Tac1* and *Tacr1* in the ARC is increased just before

480 puberty compared to earlier or later stages of postnatal development; 3) repeated exposure to

481 NK1R agonists prepubertally advances puberty onset, suggesting that the NK1R is already

482 present and functional during this developmental period. Furthermore, 4) *Tac1*KO female mice

483 exhibit a significant delay in vaginal opening [defined as complete canalization of the vagina, an

484 event that occurs with increased estrogen secretion (Caligioni 2009) and is therefore considered

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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

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509   and therefore, LH secretion (model hypothesis; Figure 1). Overall, substantial evidence exists to

510   support the hypothesis that tachykinins are indeed involved in the control of GnRH release, by

511   modulating the firing of ARC KNDy neurons either directly (NKB and SP) or indirectly (NKA) to

512   shape kisspeptin pulses (Figure 1). In addition, tachykinins, particularly SP may also act directly

513   on GnRH and/or AVPV/PeN Kiss1 neurons to contribute to: a) the shaping of GnRH pulses, and/or

514   b) the generation of the preovulatory LH surge. Many aspects of the physiology of the SP/NK1R,

515   NKA/NK2R signaling systems in the context of reproduction, remain to be fully characterized. For

516   instance, there appears to be a relative inconsistency in results between mice, rats, ruminants

517   and monkeys in the LH response to the administration of tachykinins that may reflect anatomical

518   and functional differences among species. In this regard, in humans SP is colocalized within a

519   subset of KNDy neurons (Hrabovszky, et al. 2013) whereas this is not true for all other species

520   studied to date (Kalil, et al. 2015, Navarro, et al. 2015, Rance and Bruce 1994, Rance and Young

521   1991). Furthermore, in ruminants, a much larger dose of SP is required to stimulate LH release

522   to a similar magnitude as an NKB agonist (Goodman 2015, Yamamura, et al. 2015), whereas in

523   mice, similar doses of all individual NKR agonists can lead to an increase in LH (Navarro, et al.

524   2015). However, as discussed, routes of administration, age (prepubertal versus postpubertal)

525   and sex steroid status might be a determining factor in this aspect and must be taken in to

526   account. Another important parameter that requires specific attention in future studies is the

527   considerable crossreactivity that exists between these receptor/ligand systems determining the

528   efficacy of tachykinin administration and it may be that although the three NKRs are involved in

529   the GnRH pulse generation of KNDy neurons, the ratio of the contribution of each NKR varies

530   among species and/or sexes. Nonetheless, this phenomenon may offer important advantages in

531   the treatment of disorders caused by disruption of one specific system. For example, the reversal

532   phenotype in reproductive viability observed in individuals with *TAC3/TACR3* mutations (Gianetti,

533   et al. 2010) may be due to compensation by the other tachykinin systems although this remains

534   to be elucidated. Altogether, there is a clear need for a deeper understanding of the mechanism

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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.

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