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

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12
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Contents

Introduction.....	3
The current model for the GnRH pulse generator.....	6
Anatomical studies.....	8
Distribution of SP and NKA in the hypothalamus and anatomical relationship with Kiss1 and GnRH neurons...	8
Distribution of NK1R and NK2R in the hypothalamus and anatomical relationship with Kiss1 and GnRH neurons.....	9
Sex steroid regulation of SP and NKA.....	10
Regulation of LH release by tachykinins: sex steroid dependent action.....	11
Neurokinin B.....	11
Substance P.....	12
Neurokinin A.....	14
Tachykinins modulate the gonadotropic axis in a kisspeptin dependent manner.....	15
The role of tachykinins on puberty onset.....	18
Concluding remarks.....	20

15

16 Abstract

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

33 Introduction

34 Successful production of offspring is indispensable to perpetuate species. As such, reproduction
35 is under the control of a complex regulatory network which involves the hypothalamic-pituitary-
36 gonad (H-P-G) axis. Gonadotropin-releasing hormone (GnRH) neurons, located in the
37 hypothalamus are a major component of the H-P-G axis and the ultimate regulators of
38 reproductive function, including sexual behavior (Herbison 2016, Herbison, et al. 2008, Moenter,
39 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

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-NH₂ (Maggio 1988). This family also includes
93 substance P (SP), neurokinin A (NKA), neuropeptide K (NPK), and neuropeptide γ (NP γ) (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.

122 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 (E₂)
128 and testosterone (T). By contrast, *Kiss1* expression in the AVPV/PeN—almost exclusive to the
129 female brain— is upregulated by E₂ 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

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.

163 Anatomical studies.

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

171 Distribution of SP and NKA in the hypothalamus and anatomical relationship with 172 Kiss1 and GnRH neurons.

173 Within the hypothalamus, the largest population of NKB immunoreactive cells has been detected
174 in the ARC (and specifically in the middle to caudal aspects) with smaller numbers identified in
175 the ME, POA, lateral septum, bed nucleus of the stria terminalis, amygdala and the
176 paraventricular nucleus of rats, sheep and mice (Goubillon, et al. 2000, Navarro, et al. 2009,
177 Rance and Young 1991). The ARC population has received most attention, as in this nucleus
178 kisspeptin and NKB reside in the same cell (KNDy; (Goodman, et al. 2007, Navarro, et al. 2009),
179 whereas, no instances of NKB and GnRH colocalization have been reported, although GnRH and
180 NKB immunopositive fibers have been observed to interweave in the rat ME (Krajewski, et al.
181 2005).

182 In mice, *Tac1* mRNA (encoding SP and NKA) has been mapped out in the brain of female mice
183 using *in situ* hybridization (Navarro, et al. 2015). Within the hypothalamus, expression was found
184 to be concentrated mainly in 2 regions: the ARC (especially the caudal aspect) and the
185 ventromedial nucleus (VMN), in keeping with previous reports of SP immunoreactivity in rats,
186 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.

210 [Distribution of NK1R and NK2R in the hypothalamus and anatomical relationship](#)
211 [with Kiss1 and GnRH neurons.](#)

212 Single cell RT-PCR analysis of the expression of all 3 tachykinin receptors (*Tacr1*, *Tacr2*,
213 and *Tacr3* mRNA; Table 1) in Kiss1 (ARC and AVPV/PeN) and GnRH neurons showed that
214 almost half (~49%) of Kiss1 neurons in the ARC and over one-fourth (~27%) of Kiss1 neurons in
215 the AVPV/PeN express *Tacr1* mRNA, which is also present in a subset of GnRH neurons [~23%;
216 (Navarro, et al. 2015)]. *Tacr2*, however, was absent from both populations of Kiss1 neurons and
217 GnRH neurons (Navarro, et al. 2015). Finally, *Tacr3* was confirmed to be present in all (100%)
218 ARC Kiss1 neurons but minimally present (~10%) in AVPV/PeN Kiss1 neurons, as has been
219 previously described in various species (Amstalden, et al. 2010, Navarro, et al. 2015, Navarro, et
220 al. 2009). Of note, *Tacr3* mRNA was also detected in a small subset of GnRH neurons [~11%;
221 (Navarro, et al. 2015)] as has been previously been reported in the rat (16% of GnRH somata
222 contained NK3R immunostaining) (Krajewski, et al. 2005). In addition, extensive colocalization
223 between GnRH axons with NK3R positive fibers have been reported in the ME and organum
224 vasculosum of the lamina terminalis of the rat (Krajewski, et al. 2005). Whether NK1R or NK2R
225 is expressed in KNDy and/or GnRH neurons in other species is unknown.

226 Taken together, these anatomical data allow us to postulate that SP can regulate GnRH secretion
227 indirectly, via initial action on Kiss1 neurons, but also directly by acting on GnRH neurons,
228 although functional evidence for this pathway is lacking. Furthermore, the existence of axo-axonic
229 or axo-dendritic synapses between SP and Kiss1 or GnRH axons remains to be elucidated. In
230 the human, where SP and kisspeptin have been shown to colocalize, autocrine/paracrine actions
231 of SP on KNDy neurons are also probable (Hrabovszky, et al. 2013). Intriguingly, in the mouse, a
232 subset of AVPV/PeN Kiss1 neurons are also receptive to SP actions (one fourth of these cells
233 contain NK1R) and it is well known that this population is involved in the generation of the
234 GnRH/LH surge (Oakley, et al. 2009). Therefore, a role for SP signaling in the shaping of the

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 E₂ 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),

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.

264 Regulation of LH release by tachykinins: sex steroid dependent 265 action.

266 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

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

295 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+E₂ 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+E₂ 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 + E₂ treated *versus* intact cycling monkeys. In the first study, the NK1R antagonist
329 was administered in OVX + E₂ 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

335 SP in this model. Additional detailed analysis of changes in plasma SP concentration, during the
336 periovulatory period in women showed higher SP values during the day of the LH peak, the day
337 of the descending phase and the day after the descending phase compared to all other stages in
338 the menstrual cycle (Kerdelhue, et al. 2006). However, a similar study carried out in the cycling
339 monkey, showed a decrease of plasma SP concentrations during the follicular phase leading up
340 to the LH surge and an inverse relationship between SP and estradiol values during this time
341 (Kerdelhue, et al. 2000). Thus, there appears to be a dual role for SP regarding the LH surge
342 mechanism, as there have been inhibitory and stimulatory effects reported depending on species,
343 sex steroid concentrations, as well as the timing of exposure relative to the LH surge onset. The
344 mechanism by which SP plays a role in the events leading up to the LH surge is not clear;
345 however, the fact that ~25% of Kiss1 neurons in the AVPV/PeN contain *Tac1r* provides some
346 input on a potential involvement of SP in this process (Navarro, et al. 2015). In support of this
347 notion, is the observation that SP stimulates LH to a greater extent in female compared to male
348 mice (Navarro, et al. 2015), which are devoid of an AVPV/PeV Kiss1 population (Clarkson and
349 Herbison 2006, Kauffman, et al. 2007).

350 Precedent studies on the role of SP have also reported a potential action of SP on sexual
351 behavior. The circuitry necessary for the expression of female sexual behavior, and specifically
352 the estrogen-induced display of lordosis, originates from the ventro-lateral VMN (vl VMN) and
353 projects to the midbrain periaqueductal central gray (Muntz, et al. 1980, Pfaff and Sakuma 1979,
354 Yamanouchi, et al. 1990). A number of studies have suggested that SP may be an important
355 participant in this circuitry, as SP injections in the periaqueductal central gray of OVX, estrogen-
356 primed rats produced a long-lasting increase of lordosis behavior (Dornan, et al. 1987) whereas
357 SP antiserum injections in the same region inhibit the behavior (Dornan, et al. 1987). Interestingly,
358 Fluoro-Gold injections into the dorsal midbrain labeled a large proportion (approximately 30%) of
359 the vl VMN neurons immunoreactive for SP, in the guinea pig (Ricciardi and Blaustein 1994).
360 Furthermore, pulsatile administration of estradiol, selectively induces the expression of

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 NPY. 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+E₂ 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

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

393 Tachykinins modulate the gonadotropic axis in a kisspeptin 394 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 *Kiss1* 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).

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

435 suggesting the presence of unidentified intermediate neurons upstream of Kiss1 neurons.
436 Nonetheless, even though there are still major gaps in our knowledge regarding the potential
437 mechanisms employed by each tachykinin, current data are overall, placing tachykinins in the
438 spotlight as prime candidates for the neuromodulation of kisspeptin release.

439 Despite substantial evidence for the hypothalamic action of tachykinins, we cannot ignore
440 observations that suggest a direct action of SP and NKA in the pituitary. Firstly, SP fibers have
441 been observed to surround hypophyseal portal blood capillary vessels in the ME in monkeys
442 (Kalil, et al. 2015) and NKR's have been shown to exist in pituitary cells in rats (Larsen, et al.
443 1992) and sheep (Dupre, et al. 2010). Second, it has been reported that SP and NKA can
444 stimulate LH secretion from cultured anterior pituitary cells derived from intact male rats (Kalra,
445 et al. 1992) and hemi-pituitaries (Shamgochian and Leeman 1992), respectively. These findings
446 however, are not consistent as the same was not observed in dispersed anterior pituitary cells
447 harvested from female OVX+E₂ rats (Arisawa, et al. 1990). Clearly, this pathway of action requires
448 further investigation. For example, it would be interesting to evaluate whether LH secretion is
449 stimulated after the peripheral administration of NKR agonists, but in the presence of a GnRH
450 antagonist, to rule out any central effects on, or above, GnRH neurons that these agonists might
451 exert by crossing the blood-brain barrier. This approach could potentially shed more light on the
452 likelihood of a pituitary action of tachykinins.

453 **The role of tachykinins on puberty onset.**

454 The precise neuronal and endocrine mechanisms that determine the timing of puberty onset, and
455 the subsequent achievement of reproductive capacity, remains one of the greatest unanswered
456 questions in reproductive biology. To date, several factors from central and peripheral origins
457 have been described to regulate the awakening of the gonadotropic axis (Ojeda and Lomniczi
458 2014). At a neuroendocrine level, the prevailing view is that during the infantile and juvenile

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

485 an indirect maker for puberty onset] and delayed initiation of estrous cyclicity (Simavli, et al. 2015).
486 This suggests that although E₂ 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

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

535 of action of tachykinins. We must answer: a) *whether all tachykinins participate in the generation*
536 *of LH pulses, b) if there is compensation between tachykinins to exert this role and to what extent,*
537 *c) whether the pathway (KNDy versus GnRH) of tachykinin action is governed by sex steroid*
538 *levels and the biological role of this interaction , d) if the expression of tachykinin receptors in*
539 *GnRH neurons changes (increases or decreases) in an estradiol dependent manner, e) the*
540 *anatomical relationship of tachykinins and their receptors with kisspeptin and GnRH perikarya*
541 *and fibers in other species, apart from the mouse, f) the sex and species differences in the*
542 *response to tachykinins and the contribution of SP/NK1R signaling on AVPV/PeN Kiss1 neurons*
543 *or GnRH for the occurrence of the GnRH/LH surge in the female. h) the mechanism and site of*
544 *action of NKA, as well as the phenotype of the cells that contain NK2R, which appear to be*
545 *surrogates for the indirect action of Tac1 on KNDy neurons.*

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

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562 Figure legends.

563

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

575

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