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Characterization of the role of NKA in the control of puberty onset and gonadotropin release in the female mouse.

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ABSTRACT

The tachykinin neurokinin B (NKB, *Tac2*) is critical for proper GnRH release in mammals, however, the role of the other tachykinins, such as substance P (SP) and neurokinin A (NKA) in reproduction, is still not well understood. In this study, we demonstrate that NKA controls the timing of puberty onset (similar to NKB and substance P) and stimulates LH release in adulthood through NKB-independent (but kisspeptin-dependent) mechanisms in the presence of sex steroids. Furthermore, this is achieved, at least in part, through the auto-synaptic activation of *Tac1* neurons, which express NK2R (*Tacr2*), the receptor for NKA. Conversely, in the absence of sex steroids, as observed in ovariectomy, NKA inhibits LH through a mechanism that requires the presence of functional receptors for NKB and dynorphin (NK3R and KOR, respectively). Moreover, the ability of NKA to modulate LH secretion is absent in *Kiss1*KO mice, suggesting that its action occurs up-stream of *Kiss1* neurons. Overall, we demonstrate that NKA signaling is a critical component in the central control of reproduction, by contributing to the indirect regulation of kisspeptin release.

1 Introduction

2

3 Tachykinins (TACs) are a large family of peptides that include neurokinin A and substance P
4 (NKA and SP; encoded by *TAC1*), and neurokinin B (NKB; encoded by *TAC3* or *Tac2* in rodents)
5 (1). TACs act preferentially on different G protein-coupled receptors: NK1R (encoded by *Tacr1*,
6 the receptor for SP), NK2R (encoded by *Tacr2*, the receptor for NKA) and NK3R (encoded by
7 *Tacr3*, the receptor for NKB). These TAC systems are expressed throughout the central nervous
8 system, where they participate in a variety of physiological functions, e.g. nociception and fear
9 conditioning (1,2).

10 The NKB/NK3R signaling system has emerged as a critical neuroendocrine regulator of
11 reproductive function. A growing body of evidence from our lab and others has documented the
12 stimulatory role of NKB on GnRH release, in an estradiol and kisspeptin dependent manner, in all
13 studied species including humans (3). In addition to NKB, the SP/NK1R signaling system also
14 participates in the central regulation of the gonadotropic axis, as supported by the following
15 studies: (a) the central administration of SP induces LH release in rabbits and rats (4-6), (b)
16 electrophysiology studies showed activation of Kiss1 neurons by SP (7), (c) SP mRNA and protein
17 have been found in the ARC of rodents (8,9) and (d) SP immunoreactivity has been detected
18 within Kiss1 and NKB neurons in the human infundibular nucleus (10). Interestingly, we have
19 recently shown that chronic SP administration advances puberty onset in rodents (11) and that
20 *Tac1*KO mice with congenital absence of SP display delayed puberty onset and reproductive
21 impairments (11,12). However, *Tac1* encodes both SP and NKA and thus, the reproductive
22 defects we observe in *Tac1*KO mice may be, at least in part, due to the absence of NKA signaling.
23 Importantly, we and others have documented that NKA induces LH release in mice and rats
24 (9,13,14) in a kisspeptin-dependent manner (9). Furthermore, the stimulatory action of NKA on
25 LH release is dependent on the presence of physiological levels of circulating sex steroids (9) and
26 in their absence, such as during ovariectomy (OVX), NKA inhibits LH release, similar to NKB (9).
27 However, unlike NKB, the receptor for NKA (NK2R) is not present on Kiss1 or GnRH neurons (9).
28 Therefore, we hypothesize that NKA must act upstream of Kiss1 upon an unknown population of

29 neurons, that in turn control NKB release. Alternatively, all TAC ligand-receptor systems have
30 been reported to display cross-reactivity (15), suggesting that cross-activation of NK3R could
31 account for the NKB-like action of NKA.

32 Overall, there is compelling evidence that all TACs (not only NKB) participate in the control of
33 GnRH release. Thus, deciphering TAC's individual and/or potential synergistic mechanism of
34 action, could provide important insight into the neuroendocrine control of reproduction.

35 Interestingly, a number of human patients with *TAC3/TACR3* mutations have been reported to
36 overcome initial pubertal failure and central hypogonadotropic hypogonadism (HH) (16). These
37 patients present an 'awakening' of GnRH secretion and hypogonadism reversal (16), a phenotype
38 that resembles that of *Tacr3*KO mice, which are subfertile (17). This further suggests that in the
39 absence of NKB signaling, compensation by NKA (and/or SP) may restore GnRH/LH secretion.

40 Thus, in this study, we aim to characterize the role of NKA in the control of GnRH release during
41 puberty onset and adulthood in a series of pharmacological and genetic experiments in WT,
42 *Tac2*KO and *Kiss1*KO female mice, with special interest in the interactions between the
43 NKA/NK2R and NKB/NK3R systems.

44

45 **Materials and Methods**

46 **Mice.** Wild-type (WT) female C57Bl/6 mice were purchased from Charles River Laboratories
47 International, Inc. *Tac2* KO (knockout, KO) mice were obtained from Dr. Seminara (MGH, Boston,
48 MA) (18). *Kiss1*KO were obtained from Dr. Richard Palmiter (University of Washington, Seattle,
49 WA) (19). *Tac2*KO and *Kiss1*KO mice were compared to their WT littermates. All animal studies
50 were approved by the Harvard Medical Area Standing Committee on the Use of Animals in
51 Research and Teaching in the Harvard Medical School Center for Animal Resources and
52 Comparative Medicine. Mice were maintained in a 12:12 h light/dark cycle and were fed standard
53 rodent chow diet and water ad libitum. Genotyping was conducted by PCR analyses on isolated
54 genomic DNA from tail biopsies.

55

56 **Reagents:** The agonists for NK1R (GR73632), NK2R (GR64349) and NK3R (senktide), and the
57 antagonists for NK3R (SB 222200) and NK1R (RP67580) were purchased from Tocris. Naloxone
58 Hydrochloride (opioid receptor antagonist) and GnRH were purchased from Sigma Aldrich. Mouse
59 kisspeptin-10 (Kp-10) was purchased from Phoenix pharmaceutical. All drugs were dissolved in
60 saline (0.9% NaCl), except for SB 222200 and RP67580, which were dissolved in 5% DMSO.
61 Doses and timing for hormonal analyses were selected on the basis of previous studies (9,20,21).

62

63 **Experimental design**

64 **General procedures:** For intracerebroventricular (icv) injections, the mice were briefly
65 anesthetized with isoflurane 2-3 days before the experiment and a small hole was bored in the
66 skull (1 mm lateral and 0.5 mm posterior to bregma) using a Hamilton syringe (27-gauge needle
67 fitted with polyethylene tubing, leaving 3.5 mm of the needle tip exposed). All subsequent
68 injections were made through this site. For icv injections, mice were anesthetized with isoflurane
69 for a total of 2–3 min, during which time 5 μ l of solution were slowly and continuously injected into
70 the lateral ventricle. The needle remained inserted for approximately 60 sec after the injection to
71 minimize backflow up the needle track. Mice typically recovered from the anesthesia within 3 min
72 after the injection. For hormonal analyses, blood samples (4 μ l) were obtained from the tail at 0,
73 26 and 60 min after an icv injection, and stored at -80°C until further processing. The dose and
74 time of blood sampling were selected based on our previous studies (9).

75

76 **Study 1: *Effect of chronic NK2R-Ag administration in pre-pubertal mice.***

77 To investigate whether NKA/NK2R signaling plays a role in puberty onset, we performed a
78 systemic chronic (from 23d to 32d) administration of NK2R-Ag (3 nmol/100 μ l/i.p./every 12h) or
79 vehicle (0.9% NaCl) to WT female mice ($n \geq 6$ per group). Reproductive maturation (i.e.,
80 progression of vaginal opening; VO) was monitored daily. Body weight (BW) was recorded at day
81 30 when 50% of control females showed VO. Lastly, uterine and ovarian weights, as well as LH
82 concentrations, were determined at day 32, the final day of NK2R-Ag administration.

83 **Study 2:** *Interaction between NKA, NKB and SP for the stimulation of LH release in the presence*
84 *of estradiol.*

85 In this study, adult WT female mice were subjected to bilateral OVX under light isofluorane
86 anesthesia, 1 week before pharmacological tests. Immediately after OVX, capsules filled with
87 diluted crystalline of 17 β -estradiol (E₂) or vehicle (sesame seed oil) were implanted
88 subcutaneously (sc) via a small midscapular incision at the base of the neck (OVX+E₂). Silastic
89 tubing (15 mm long, 0.078 in inner diameter, 0.125 in outer diameter; Dow Corning) was used for
90 capsule preparation. A low dose of crystalline E₂ (50 μ g/mL, in sesame oil) was used to fill the
91 capsules which were sealed with silicone cement and allowed to cure overnight. The day before
92 surgery, implants were washed twice for 10 min in changes of 100% ethanol and then placed in
93 sterile physiological saline overnight.

94 First, we aimed to investigate the potential additive effect of the NK2R-Ag and senktide on LH
95 secretion in the presence of sex steroids. To this end, LH levels were measured in WT OVX+E₂
96 females (n \geq 5 per group), 25 and 60 min after an icv injection of NK2R-Ag (600pmol), senktide
97 (600pmol) or the co-administration of both drugs. Next, WT OVX+E₂ females (n \geq 5 per group)
98 were pretreated with the NK3R antagonist SB222200 (7 nmol), 60 minutes prior to the icv injection
99 of NK2R-Ag (600 pmol, senktide or vehicle (0.9% NaCl). Blood samples were collected before
100 SB222200 injection (basal) and at 25 and 60 minutes after injection of the agonists. Additionally,
101 we further investigated the action of NK2R-Ag in the absence of NKB signaling using *Tac2*KO
102 OVX+E₂ females and their corresponding WT littermate controls (WT OVX+E₂; n \geq 5 per group).
103 Both groups of females were injected with NK2R-Ag (600 pmol) and blood samples were collected
104 before and 25 and 60 min after injection. Finally, in order to evaluate whether the action of NKA
105 requires kisspeptin to stimulate LH release, we used *Kiss1*KO OVX+E₂ females and their
106 corresponding WT littermate controls (WT OVX+E₂; n \geq 5 per group) and LH levels were measured
107 25 min after icv injection of NK2R-Ag (600 pmol).

108

109 **Study 3:** *Interaction between NKA, NKB and SP for the inhibition of LH release in*
110 *ovariectomized female mice.*

111 Adult WT females were subjected to bilateral OVX under light isoflurane anesthesia 1 week
112 before pharmacological tests. First, we aimed to investigate the potential additive effect of NK2R-
113 Ag and senktide in the inhibition of LH secretion in the absence of sex steroids. Thus, LH levels
114 were measured in WT OVX females (n≥5 per group), 25 and 60 min after an icv injection of NK2R-
115 Ag (600pmol), senktide (600pmol) or the co-administration of both drugs. In the next experiment,
116 we intended to assess the role of NKB in the inhibition of LH secretion achieved by NKA, in WT
117 OVX females. To this end, LH responses to the NK2R-Ag were evaluated after blockade of the
118 effects of NKB using SB222200 (7 nmol) as a selective antagonist for NK3R. For this purpose,
119 adult WT OVX female mice (n≥5 per group) were pretreated with SB222200 60 minutes prior to
120 the icv injection of NK2R-Ag (600 pmol). Blood samples were collected before SB222200 injection
121 (basal) and at 25 and 60 minutes after vehicle or NK2R-Ag injection. In addition, we investigated
122 the role of endogenous opioids in the control of LH secretion and in the modulation of LH
123 responses to NK2R, using adult WT OVX females. To this end, LH responses to NK2R-Ag were
124 measured after the blockade of the κ and μ opioid receptors (KOR and MOR) using naloxone
125 (5mg/kg/100ul/ip). All animals (WT OVX females; n≥5 per group) were injected with naloxone, 12
126 hours and then 60 minutes prior to the icv injection of NK2R-Ag (600 pmol). Blood samples were
127 collected before naloxone injection and at 25 and 60 minutes after NK2R-Ag injection. We further
128 investigated the action of NK2R-Ag in the absence of NKB signaling and sex steroids using
129 *Tac2*KO OVX and WT OVX females (n≥5 per group). Both groups of animals were injected with
130 NK2R-Ag (600 pmol) and blood samples were collected before and then 25 and 60 min after
131 injection. In addition, we evaluated the role of SP in the NK2R-Ag induced inhibition of LH in WT
132 OVX and *Tac2*KO OVX mice (n≥5 per group). Both groups of females were injected with NK1R-
133 Antg (2 nmol), 30 min before the administration of NK2R-Ag (600 pmol), NK1R-Ag (600 pmol) or
134 vehicle. Blood samples were collected before and then 25 and 60 min after injection. Finally, we
135 assessed the ability of NK2R signaling to modulate LH release in the absence of kisspeptin and

136 sex steroids using *Kiss1*KO OVX and WT OVX females (n≥5 per group), which were injected with
137 NK2R-Ag (600 pmol) and LH levels were measured at 25 and 60 min after icv injection.

138

139 **Study 4: Expression of *Tacr1*, *Tacr2*, *Tacr3*, *Kiss1* and *Pdyn* in the mediobasal hypothalamus**
140 *(MBH) of female mice.*

141 We aimed to determine if there are changes in the expression of *Tacr1*, *Tacr2*, *Tacr3*, *Kiss1*, and
142 *Pdyn* in the mediobasal hypothalamus (MBH), the site that includes the arcuate nucleus (ARC)
143 between WT (n = 7) and *Tacr2* KO (n = 9) ovary-intact females.

144 Total RNA from the MBH was isolated using TRIzol reagent (Invitrogen) followed by
145 chloroform/isopropanol extraction. RNA was quantified using NanoDrop 2000 spectrophotometer
146 (Thermo Scientific), and 1 µm of RNA was reverse transcribed using iScript cDNA synthesis kit
147 (Bio-Rad). Quantitative real-time PCR assays were performed on an ABI Prism 7000 sequence
148 detection system, and analyzed using ABI Prism 7000 SDS software (Applied Biosystems). The
149 cycling conditions were the following: 2 min incubation at 95°C (hot start), 45 amplification cycles
150 (95°C for 30 s, 60°C for 30 s, and 45 s at 75°C, with fluorescence detection at the end of each
151 cycle), followed by melting curve of the amplified products obtained by ramped increase of the
152 temperature from 55 to 95°C to confirm the presence of single amplification product per reaction.
153 For data analysis, relative standard curves were constructed from serial dilutions of one reference
154 sample cDNA and the input value of the target gene was standardized to *Hprt* levels in each
155 sample. The primers used are listed in **Table 1**.

156

157 **In situ hybridization (ISH):** To determine the presence of co-expression between *Tacr2r* and
158 *Tacr1* mRNA in key areas (ventromedial nucleus, VMN; and ARC), dual fluorescence ISH was
159 performed in tissue samples from OVX+sham and OVX+E₂ mice. We used probes for *Tacr2r*-C1
160 and *Tacr1*-C2 obtained from ACDBio and used the RNAscope method per their protocol (ACDBio).
161 The brains were removed for ISH, fresh frozen on dry ice, and then stored at -80°C until
162 sectioning. Five sets of 20-µm sections in the coronal plane were cut on a cryostat, from the

163 diagonal band of Broca to the mammillary bodies, thaw mounted onto SuperFrost Plus slides
164 (VWR Scientific), and stored at -80°C . A single set was used for ISH experiment (adjacent
165 sections 100 μm apart).

166

167 **Hormone measurements:** LH was measured by a sensitive sandwich ELISA for the assessment
168 of whole blood LH concentrations (22). A 96-well high-affinity binding microplate (9018; Corning)
169 was coated with 50 μL of capture antibody (monoclonal antibody, anti-bovine LH beta subunit,
170 518B7; University of California) at a final dilution of 1:1000 (in 1XPBS, 1.09 g of Na_2HPO_4 [an-
171 hydrous], 0.32 g of NaH_2PO_4 [anhydrous], and 9g of NaCl in 1000 mL of distilled water) and
172 incubated overnight at 4°C . To minimize unspecific binding of the capture antibody, wells were
173 incubated with 200 μL of blocking buffer (5% [w/v] skim milk powder in 1XPBS-T (1XPBS with
174 0.05% Tween20) for 2 hours at room temperature (RT). A standard curve was generated using a
175 2-fold serial dilution of LH (reference preparation, AFP-5306A; National Institute of Diabetes and
176 Digestive and Kidney Diseases National Hormone and Pituitary Program [NIDDK-NHPP]) in 0.2%
177 (w/v) BSA-1XPBS-T. The LH standards and blood samples were incubated with 50 μL of detection
178 antibody (polyclonal antibody, rabbit LH antiserum, AFP240580Rb; NIDDK-NHPP) at a final
179 dilution of 1:10000 for 1.5 hours (at RT). Each well containing bound substrate was incubated
180 with 50 μL of horseradish peroxidase conjugated antibody (polyclonal goat anti-rabbit, D048701–
181 2; DakoCytomation) at a final dilution of 1:2000. After a 1.5-hour incubation, 100 μL of o-
182 phenylenediamine (002003; Invitrogen), substrate containing 0.1% H_2O_2 was added to each well
183 and left at RT for 30 minutes. The reaction was stopped by addition of 50 μL of 3M HCl to each
184 well, and absorbance of each well was read at a wave length of 490 nm (Sunrise; Tecan Group).
185 The concentration of LH in whole blood samples was determined by interpolating the OD values
186 of unknowns against a nonlinear regression of the LH standard curve (22).

187

188 **Statistical Analysis:** All data are expressed as the mean \pm SEM for each group. A two tailed
189 unpaired t-Student test or a one- or two-way ANOVA test followed by Tukey or Newman Kleus

190 *post-hoc* test was used to assess variation among experimental groups. Significance level was
191 set at $P < 0.05$. All analyses were performed with GraphPad Prism Software, Inc (San Diego, CA).

192

193 **Author contributions**

194 SL and VMN conceived and designed the research. SL, CF, RT, SS, CAM and AG conducted
195 experiments. SL and VMN contributed to data analysis. SL and VMN wrote the manuscript, and
196 all authors contributed to manuscript editing.

197

198

199 **Results**

200

201 **1. Advancement of puberty onset after chronic activation of NK2R in female mice.**

202 Our previous studies have demonstrated that chronic administration of specific agonists for the
203 NK1R and NK3R receptor are able to advance puberty onset in mice and rats (11,20), indicating
204 that these systems are in place before puberty and likely participate in the proper timing of puberty
205 onset. However, whether NKA/NK2R signaling is also involved in the awakening of the
206 gonadotropic axis at the time of puberty is unknown. To address this question, we chronically
207 (every 12h) treated WT females with a specific agonist of NK2R from weaning age (22d) to 32d.
208 We observed that this treatment was able to advance puberty onset as evidenced by the
209 advanced timing of VO and increased uterine and ovarian weight compared to controls [uterine
210 weight: 19.55 ± 1.89 mg *versus* 23.79 ± 1.61 mg in control and NK2R-Ag treated females,
211 respectively ($*p < 0.05$) and ovarian weight: 8.3 ± 0.56 mg *versus* 11.7 ± 0.44 mg in control and
212 NK2R-Ag treated females, respectively ($***p < 0.001$); **Figure 1 A-F**]. BW at 30d was not different
213 between groups.

214

215 **2. The receptor for NKA (NK2R) is expressed in VMH *Tac1* neurons.**

216 We have previously documented the existence of mRNA for the NKB receptor (NK3R) and SP
217 receptor (NK1R) in Kiss1 neurons in the ARC, while the receptor for NKA (NK2R) was
218 undetectable in Kiss1 or GnRH neurons (9). We therefore aimed to assess if NK2R (encoded by

219 *Tacr2*) is expressed in other neurons located in the ARC or ventromedial hypothalamus (VMH)
220 and whether it colocalizes with *Tac1* neurons in these areas as the source of SP and NKA. Our
221 *in situ* hybridization (RNAscope) results showed that *Tacr2* is expressed in both ARC and VMH
222 nuclei and colocalizes with virtually all *Tac1* neurons in the VMH of adult WT mice regardless of
223 the E₂ milieu (**Figure 2**).

224

225 **3. The stimulatory action of NKA is independent of NKB but dependent of kisspeptin.**

226 We previously reported that the action of NKA and NKB on LH release is largely equivalent, i.e.
227 both increase LH release in the presence of physiological circulating levels of E₂, but inhibit LH in
228 the absence of sex steroids (9). It was, therefore, tentative to speculate that NKA could induce
229 LH release through the stimulation of NKB given the absence of NK2R in *Kiss1* and GnRH
230 neurons. To test this hypothesis, first we co-administered NK2R and NK3R agonists in OVX + E₂
231 WT mice and observed that the increase in LH was similar in groups injected with an individual
232 dose of each agonist or the combination of both (**Figure 3 A**), eliminating the possibility of an
233 additive effect of NKA and NKB on LH release and suggesting a possible common pathway. Next,
234 to evaluate if NKA requires NKB signaling to induce LH release, the LH response to NK2R-Ag
235 was tested in the presence of an NK3R antagonist (**Figure 3 A**) or in NKB deficient (*Tac2KO*)
236 mice (**Figure 3 B**). In both cases, NK2R-Ag was able to significantly stimulate LH release
237 indicating that NK2R activation induces LH release independently of the presence of NKB or its
238 receptor NK3R. However, NK2R signaling requires the presence of kisspeptin as *Kiss1KO* mice
239 replaced with E₂ did not have any effect on LH release (WT Basal: 0.27 ± 0.06 ng/mL, WT NK2R-
240 Ag: 0.57 ± 0.09 ng/mL; *p < 0.05; *Kiss1KO* Basal: 0.21 ± 0.02 ng/mL, *Kiss1KO* NK2R-Ag: 0.29
241 ± 0.04 ng/mL; not significant).

242

243 **4. The inhibitory action of NKA is NKB and dynorphin dependent.**

244 In the next set of experiments, we sought to determine whether the inhibitory action of NKA/NK2R
245 on LH release in the absence of sex steroids (i.e. OVX) is mediated by NKB or dynorphin. First,

246 we showed that the inhibitory action of NK2R-Ag + senktide was similar to that of senktide alone,
247 suggesting (as in the presence of E₂) that there is no additive effect of both tachykinins in the
248 inhibition of LH (**Figure 4 A**). The use of the specific NK3R antagonist alone decreased LH in
249 OVX animals, in line with recent literature showing that blockade of NK3R decreases LH pulsatility
250 (23-26). However, co-administration of the NK3R antagonist and the NK2R-Ag failed to induce a
251 further decrease in LH, suggesting that NK3R signaling is required for the inhibitory action of NKA
252 in the absence of E₂ (**Figure 4 A**). Moreover, as previously described in rats, NK2R signaling
253 requires dynorphin to inhibit LH (27), which is prevented after the blockade of the KOR and MOR
254 using naloxone (**Figure 4 B**). Of note, naloxone alone also inhibited LH release in OVX mice, in
255 line with our previous reports in OVX *Pdyn*KO and *Oprk1*KO mice (dynorphin KO and KOR KO
256 mice, respectively) (28), suggesting that the absence of the inhibitory signal of dynorphin leads to
257 a significant decrease in the ability of the mouse to secrete LH, probably due to disruption of the
258 LH pulse generator mechanism (29). Next, we assessed the action of NK2R-Ag in the congenital
259 absence of NKB (OVX *Tac2*KO mice) to further confirm the data obtained after NK3R blockade.
260 Unexpectedly, we observed that the absence of NKB leads to a robust induction of LH release
261 (**Figure 5 A**), revealing an action that is not present in WT OVX regardless of whether a functional
262 NK3R is present or antagonized.

263 Because we have observed that VMH Tac1 neurons co-express *Tacr2* (NK2R) (**Figure**
264 **2**), we hypothesized that NKA could be inducing LH release in *Tac2*KO mice through the
265 stimulation of SP from Tac1 neurons that, in turn, would activate Kiss1 neurons (9). To test this
266 hypothesis, we administered the NK2R-Ag in the presence of a NK1R antagonist (**Figure 5 A**)
267 [proven to efficiently block the action of a NK1R- agonist (**Figure 5 B**)] in *Tac2*KO OVX mice.
268 NK2R-Ag was still able to induce LH release after NK1R blockade although the magnitude of this
269 increase tended to be lower than in the absence of the NK1R antagonist (**Figure 5 A**). Lastly, we
270 confirmed that this action is kisspeptin-dependent by showing complete absence of LH response
271 after the administration of NK2R-Ag in *Kiss1* KO mice (WT: Basal 2,90 ± 0.42 ng/mL, NK1R-Ag
272 2.08 ± 0.32 ng/mL, *p < 0.05; *Kiss1*KO: Basal 0.32 ± 0.06 ng/mL, NK1R-Ag 0.30 ± 0.02 ng/mL,

273 not significant). In order to assess whether this striking difference between the response to NK2R
274 agonists after the pharmacological blockade of NK3R and the congenital absence of the NK3R
275 ligand (NKB) is due to compensation in the expression of any of the ligand-receptor components
276 of the tachykinin systems or in dynorphin, we evaluated the expression of these genes in the MBH
277 of WT vs. *Tac2KO* female mice. We observed a significant increase in the expression of *Tacr3*
278 (NK3R) in *Tac2KO* mice compared to controls (**Figure 5 C**).

279

280 **Discussion**

281 The neuroendocrine mechanisms controlling the timing of puberty onset remain largely
282 unknown. Among the stimulatory signals that increase their synthesis and release in the late
283 juvenile period to induce the awakening of the reproductive axis, kisspeptin plays a pivotal role
284 (30). Inactivating mutations in the *KISS1/KISS1R* genes lead to HH and absent puberty onset
285 (31,32), while gain of function mutations in *KISS1R* advances puberty onset in humans (33).
286 Similarly, chronic administration of kisspeptin-10 advances puberty onset in rodents (34).
287 However, the pattern of kisspeptin release is dependent on upstream regulators, such as the
288 tachykinin peptides (ref). For example, the tachykinin NKB, acts autodynamically in ARC Kiss1
289 neurons, and its activation precedes that of kisspeptin, to allow for the proper timing of puberty
290 onset (28,35-38). Moreover, we have recently documented that the tachykinin SP, originating
291 from *Tac1* neurons and located upstream of *Kiss1*, is also involved in puberty initiation (11,12). In
292 this study, we expand these findings to include NKA, and demonstrate that prepubertal female
293 mice exhibit a premature activation of the reproductive axis in response to this tachykinin. , chronic
294 activation of the NKA receptor (NK2R) during this developmental period, advances puberty onset
295 in female mice, as observed by the advanced age of vaginal opening and increased ovarian and
296 uterine weights.. While this finding demonstrates that the animal is able to respond to the
297 stimulatory action of NKA prepuberally, further studies will be required to determine the
298 contribution of NKA on the timing of puberty onset. However, this finding suggests that the delay

299 in puberty onset observed in *Tac1*KO mice (11) may be due to the loss of the stimulatory action
300 of both SP and NKA on kisspeptin release.

301 Interestingly, while the vast majority of ARC Kiss1 neurons express NK3R (*Tacr3*) and
302 approximately half express NK1R (*Tacr1*), no detectable expression of NK2R (*Tacr2*) has been
303 found in Kiss1 neurons or GnRH neurons (9). This suggests that the primary action of NKA must
304 lie upstream of Kiss1 neurons. In this study, we identified *Tacr2* in the majority (x%) of *Tac1*
305 neurons located in the VMH. Whether this population of *Tac1* neurons is the main source of NKA
306 that elicits gonadotropin release is still unclear and will require the use of genetic mouse and viral
307 models. Nonetheless, the high degree of colocalization between the ligand (NKA encoded by
308 *Tac1*) and its receptor (*Tacr2*), as observed in the VMH, is reminiscent of the NKB/NK3R
309 signaling mechanism in ARC Kiss1 neurons and suggests the existence of an aut synaptic loop
310 that may modulate the release of SP onto Kiss1 neurons. Of note, a scarce population of *Tac1*
311 neurons is also present in the ARC, and adjacent to Kiss1 neurons (9). However, these neurons
312 do not express *Tacr2* and their role, if any, in the control of Kiss1 neurons remains to be
313 characterized.

314

315 In this study we also addressed the question of whether the analogous action of NKA and NKB
316 in the regulation of LH release (i.e. stimulation in the presence of E₂ and inhibition in its absence
317 (9)) is due to the convergence of the NKA mechanism of action onto NKB signaling. Despite the
318 aforementioned functional similarities, our data using an NK3R antagonist and *Tac2*KO mice after
319 OVX or OVX and E₂ replacement, clearly demonstrate that the stimulatory action of NKA on LH
320 is NKB *independent* but kisspeptin-dependent, suggesting the existence of a yet unknown
321 population of NKA-responsive neurons that in turn activate Kiss1 neurons to induce
322 kisspeptin/GnRH release (**Figure 6**). In contrast, NKA has been shown to inhibit LH via a
323 mechanism which involves dynorphin in the rat (14,27) similar to what has been described for
324 NKB in the absence of E₂ (39). Here, we show that the inhibitory action of NKA is weaker than the
325 one exerted by NKB as observed 60 min after treatment, when NK2R agonist's action on the

326 inhibition of LH release is lost, while this inhibition reaches a maximal level for the NK3R agonist.
327 Furthermore, we demonstrate that the inhibitory action of NKA is mediated by the activation of
328 the NKB–dynorphin signaling pathway since blockade of both NK3R and KOR receptors
329 prevented the inhibition of LH induced by the NK2R agonist. Importantly, the action of stimulating
330 or inhibiting NK3R and KOR in both cases leads to the inhibition of LH release in the absence of
331 sex steroids that is of equal magnitude 25 min after the treatment, likely as a consequence of the
332 disruption of the GnRH pulse generator at the level of the ARC Kiss1 neuron. However, we
333 unexpectedly observed that in the congenital absence of NKB, the NK2R agonist significantly
334 stimulates LH release in the absence of E₂ (our present data in *Tac2KO* mice). These data
335 suggest that the action of NKA is inherently stimulatory, NKB-independent and kisspeptin-
336 dependent, as NK2R agonists did not induce any LH release in *Kiss1KO* mice regardless of the
337 sex steroid milieu, unlike our recent findings on the kisspeptin-independent action of NKB (19).
338 Thus, in *Tac2KO* mice, where NK3R and KOR are not blocked, the activity of the ARC Kiss1
339 neuron is significantly lower due to the absence of the stimulatory action of NKB, leading to lower
340 basal LH levels after OVX (unpublished data and Figure 5). In this scenario, NKA is able to further
341 stimulate Kiss1 neurons, leading to an increase in LH release. Whether this reflects
342 heterodimerization of NK3R with NK1R, as we previously reported (9), heterodimerization with
343 other receptors, or the convergence of intracellular pathways with NK3R's, remains unknown.
344 Interestingly, the congenital absence of *Tac2* led to the compensatory rise of *Tacr3* and a
345 noticeable trend to increase *Tacr1*. This is reminiscent of the increase in *Tacr2* observed in the
346 absence of *Tac1* (*Tac1KO* mice (11)) and could also account, at least partially, for the increase
347 in LH by NK2R agonists in *Tac2KO* mice regardless of the sex steroid milieu.

348 Altogether, these data suggest that NKA contributes to the activation of ARC Kiss1 neurons
349 through a process that may involve auto-synaptic signaling on VMH *Tac1* neurons to induce SP
350 release (as observed by the lower induction of LH release in the presence of a NK1R-antagonist),
351 as well as the activation of yet unknown NKA-responsive neurons that eventually further regulate
352 ARC Kiss1 neurons (**Figure 6**).

353

354 Overall, in this study we have demonstrated that NKA is able to advance the timing of puberty
355 onset in females, along with SP, NKB and kisspeptin. Moreover, it offers new insights into the
356 interaction and mechanism of action of tachykinins in the control of LH release, especially related
357 to NKA-NKB interaction, which remained largely unexplored. Importantly, this study suggests that
358 in the absence of NKB, the derived hypogonadism could be compensated (and potentially
359 reversed) by NKA, which may account for the reversal of the HH phenotype frequently observed
360 in *TAC3* deficient patients. Thus, the exogenous activation of the NKA signaling pathway may
361 offer a novel approach for treating these patients in the clinic.

362

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- 490

491

492 **Figure legends**

493 **Figure 1. Advancement of puberty onset after chronic activation of NK2R in female mice.**

494 Repeated stimulation (every 12 h) of WT female mice with GR64349 (NK2R-A, 3 nmol/100ul/ip)
495 or vehicle (0.9% NaCl/100ul/ip) from p23 to p32 (n≥6 per group). **(A)** Progression of VO, **(B)** mean
496 postnatal day of VO and **(C)** BW the day of 50% of the control animals displayed VO. **(D)** Uterine
497 weight, **(E)** ovarian weight and **(F)** serum LH levels at p32. Statistical analysis was performed
498 using a 2-tailed t test (*p < 0.05; ***p< .001).

499

500 **Figure 2. *Tacr2* (NK2R) is expressed in VMH *Tac1* neurons.**

501 Representative double label ISH depicting co-localization of *Tac1* (red) and *Tacr2* (green) mRNA.
502 **(A)** VMH and **(C)** ARC of female WT C57Bl/6 mice after 1 week of OVX. **(B)** VMH and **(D)** ARC
503 of female WT C57Bl/6 OVX mice after 1 week of E₂ replacement.

504

505 **Figure 3. The stimulatory action of NKA is independent of NKB in the presence of** 506 **physiological circulating levels of E₂.**

507 **(A)** LH release before (basal), 25 and 60 min after the icv injection of NK2R-Ag, senktide or the
508 co-administration of both (600 pmol/5ul/icv) in WT OVX+E₂ females (n ≥ 5 per group). *p<0.05
509 vs. corresponding basal levels. (2 Way ANOVA followed by Tukey *post hoc* test). For SB222200
510 treated mice, LH levels before (basal) SB222200 (7 nmol/5ul/icv) administration and at 25 and 60
511 minutes after injection of NK2R-Ag (600 pmol/5ul/icv), senktide (600 pmol/5ul/icv) or vehicle
512 (0.9% NaCl/5ul/icv) in WT OVX+E₂ females (n ≥ 4 per group). *p<0.05 vs. corresponding basal

513 levels. # $p < 0.05$ vs. corresponding control mice at the same time point (2 Way ANOVA followed
514 by Tukey *post hoc* test). **(B)** LH before (Basal) and at 25 and 60 minutes after injection of NK2R-
515 Ag (600 pmol/5ul/icv) in OVX+E₂ WT and *Tac2KO* females ($n \geq 5$ per group). * $p < 0.05$ vs.
516 corresponding basal levels (2 Way ANOVA followed by Tukey *post hoc* test).

517

518 **Figure 4. The inhibitory action of NKA is NK3R and KOR dependent.**

519 **(A)** LH release before (basal), 25 and 60 min after icv injection of NK2R-Ag, senktide or the co-
520 administration of both (600 pmol/5ul/icv) in WT OVX females ($n \geq 5$ per group). * $p < 0.05$ vs.
521 corresponding basal levels; # $p < 0.05$ vs. NK2R-Ag at the same time point (2 Way ANOVA
522 followed by Tukey *post hoc* test). For SB222200 treated mice, LH levels before (basal) SB222200
523 (7 nmol/5ul/icv) injection and at 25 and 60 minutes after injection of NK2R-Ag (600 pmol/5ul/icv),
524 senktide (600 pmol/5ul/icv) or vehicle (0.9% NaCl/5ul/icv) in WT OVX females ($n \geq 5$ per group).
525 * $p < 0.05$ vs. corresponding basal levels (2 Way ANOVA followed by Tukey *post hoc* test). **(B)** LH
526 levels before (basal), 25 and 60 minutes after injection of naloxone (5mg/kg/100ul/ip) or vehicle
527 (0.9% NaCl/100ul/ip) in WT OVX females ($n \geq 5$ per group). * $p < 0.05$ vs. corresponding basal levels
528 (2 Way ANOVA followed by Tukey *post hoc* test).

529

530 **Figure 5. The inhibitory action of NKA is NKB independent and partially SP dependent.**

531 **(A)** LH levels before (basal) (vehicle or NK1R-Antg (2 nmol/5ul/icv)) and at 25 and 60 minutes
532 after injection of NK2R-Ag (600 pmol/5ul/icv) or vehicle (0.9% NaCl/5ul/icv) in OVX *Tac2KO*
533 females ($n \geq 5$ per group). * $p < 0.05$ vs. corresponding basal levels (2 Way ANOVA followed by
534 Tukey *post hoc* test). **(B)** LH levels before (basal) (vehicle or NK1R-Antg (2 nmol/5ul/icv)) and at
535 25 and 60 minutes after injection of NK1R-Ag (600 pmol/5ul/icv), or vehicle (0.9% NaCl/5ul/icv)
536 in WT OVX females ($n \geq 5$ per group). * $p < 0.05$ vs. corresponding basal levels (2 Way ANOVA
537 followed by Tukey *post hoc* test); # $p < 0.05$ vs. NK1R-Antg + vehicle injected mice levels. **(C)**
538 Expression of *Tac1r*, *Tacr2*, *Tacr3*, *Kiss1* and *Pdyn* in the mediobasal hypothalamus of *Tac2KO*
539 females ($n=9$) and their WT controls ($n=7$). * $p < 0.05$ Student t-test.

540 **Figure 6. Schematic representation of the proposed mechanism of action of NKA.**

541 NKA, expressed by Tac1 neurons in the VMH, regulates the activity of ARC Kiss1 neurons
542 through two potential mechanisms: 1) through autosynaptic loops that regulate the release of SP
543 onto Kiss1 neurons and 2) through the action of NKA on nearby (unidentified) NKA-responsive
544 neurons that eventually contact Kiss1 neurons.