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1 **Tachykinin signaling is required for the induction of the preovulatory LH surge and normal LH**  
2 **pulses.**

3  
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43 **ABSTRACT**

44 Tachykinins (NKA, NKB and Substance P) are important components of the neuroendocrine  
45 control of reproduction by directly stimulating Kiss1 neurons to control GnRH pulsatility, essential  
46 for reproduction. Despite this role of tachykinins for successful reproduction, knockout mice for  
47 *Tac1* (NKA/SP) and *Tac2* (NKB) genes are fertile, resembling the phenotype of human patients  
48 bearing NKB signaling mutations, who often reverse their hypogonadal phenotype. This suggests  
49 the existence of compensatory mechanisms among the different tachykinin ligand-receptor  
50 systems, to maintain reproduction in the absence of one of them. In order to test this hypothesis,  
51 we generated complete tachykinin deficient mice (*Tac1/Tac2*KO). Male mice displayed delayed  
52 puberty onset and decreased LH pulsatility (frequency and amplitude of LH pulses) but preserved  
53 fertility. However, females did not show signs of puberty onset (first estrus) within 45 days after  
54 vaginal opening, displayed low frequency (but normal amplitude) of LH pulses and 80% of them  
55 remained infertile. Further evaluation identified a complete absence of the preovulatory LH surge  
56 in *Tac1/Tac2*KO females as well as in WT females treated with NKB or SP receptor antagonists.  
57 These data confirmed a fundamental role for tachykinins in the timing of puberty onset and LH  
58 pulsatility and uncovered a role of tachykinin signaling in the facilitation of the preovulatory LH  
59 surge. Overall, these findings indicate that tachykinin signaling plays a dominant role in the control  
60 of ovulation, with potential implications as pathogenic mechanism and therapeutic target to  
61 improve reproductive outcomes in women with ovulation impairments.

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## 77 INTRODUCTION

78 Tachykinins are a family of peptides comprised of neurokinin A (NKA) and substance P  
79 (SP), encoded by *Tac1*, and neurokinin B (NKB), encoded by *Tac2* (*TAC3* in humans)<sup>1</sup>.  
80 Inactivating mutations in the *TAC3* gene and in the gene encoding NKB's receptor NK3R (*TACR3*)  
81 leads to hypogonadotropic hypogonadism (HH) and infertility in human patients<sup>2,3</sup>. NKB is  
82 expressed in Kiss1 neurons of the arcuate nucleus (Kiss1<sup>ARC</sup>), which express kisspeptin, the most  
83 potent secretagogue of GnRH identified to date<sup>4,5</sup>. Our lab and others have contributed to an  
84 extensive body of literature that supports a predominantly stimulatory role of NKB on the  
85 reproductive axis acting as a regulator of kisspeptin release to stimulate GnRH pulses<sup>1</sup>. This role  
86 is supported by the infertile phenotype of human patients bearing *TAC3/TACR3* mutations.  
87 Because *Tac1* is not expressed in Kiss1 neurons but upstream of these<sup>6</sup>, and despite the  
88 documented stimulatory effect of SP<sup>6-13</sup> and NKA<sup>6,14,15</sup> on LH release, no inactivating mutations in  
89 this gene have been reported as causative of hypogonadotropic hypogonadism (HH) in humans,  
90 to date. This indicates that the absence of NKA/SP signaling can be compensated by alternative  
91 neurocircuitries and/or that their role is merely to fine-tune kisspeptin release. Interestingly,  
92 despite this critical role of NKB, a number of NKB signaling deficient patients reverse their  
93 hypogonadal phenotype<sup>16</sup>, being able to have successful pregnancies before relapsing into their  
94 HH state again. This suggests that under specific circumstances, NKB signaling can be  
95 compensated leading to the temporary activation of the reproductive axis. In fact, this phenotype  
96 is in line with that observed in mouse models devoid of NKB signaling (*Tac2*KO and *Tacr3*KO)<sup>17,18</sup>  
97 and NKA/SP signaling (*Tac1*KO)<sup>11,19</sup>. These mouse models present delayed puberty onset but  
98 overall preserved fertility. Importantly, although SP and NKB have high affinity for NK1R and  
99 NK3R receptors, respectively; NKB can also bind NK1R and similarly, SP can activate NK3R,  
100 suggesting a high degree of redundancy in the tachykinin system with strong likelihood of  
101 compensation in the absence of one of the ligand/receptor systems<sup>20,21</sup>. We hypothesized that  
102 this degree of redundancy is the answer to the reversal of the HH phenotype observed in

103 NKB/NK3R null patients, as well as the reason for the preserved fertility in each of the individual  
104 KO mouse models. In order to assess this hypothesis, we generated a complete tachykinin null  
105 mouse model (*Tac1/Tac2* KO) and complete reproductive characterization was performed in both  
106 sexes.

107

## 108 **MATERIALS AND METHODS**

109 **Mice.** *Tac2* KO (knockout, KO) mice were obtained from Dr. Seminara (MGH)<sup>17</sup>. *Tac1/Tac2* KO  
110 were generated by crossing *Tac1*KO (The Jackson Laboratories, stock No. 004103) and *Tac2*KO  
111 mice. All animal studies were approved by the Brigham and Women's Hospital Institutional Animal  
112 Care and Use Committee. Mice were maintained in a 12:12 h light/dark cycle and were fed  
113 standard rodent chow diet and water *ad libitum*. Genotyping was conducted by PCR analyses on  
114 isolated genomic DNA from tail biopsies.

115

116 **Reagents:** The antagonists of NK3R (SB 222200) and NK1R (RP67580) were purchased from  
117 Tocris Bioscience (Minneapolis, MN). Doses and timings for hormonal analyses were selected on  
118 the basis of previous studies<sup>6,22,23</sup>.

119

## 120 **Experimental design**

121 **Study 1:** *Reproductive maturation of Tac1/Tac2 KO male and female mice.*

122 In order to assess the reproductive phenotype of mice lacking all of the tachykinins, and therefore,  
123 prevent cross reactivity of their ligand-receptor systems that potentially compensates for the  
124 absence of one of them, we generated a double *Tac1* and *Tac2* KO mouse (*Tac1/Tac2*KO).  
125 Prepubertal littermate WT (n=11) and *Tac1/Tac2* KO (n=14) males were monitored daily from  
126 postnatal 25d for preputial separation as an indirect marker of puberty onset. Body weight was  
127 measured at the average age of puberty onset (28d).

128 In females, littermate WT (n=12) and *Tac1/Tac2* KO (n=8) were monitored daily from postnatal  
129 25d for body weight (BW) and pubertal progression (vaginal opening [VO] as indicated by  
130 complete canalization of the vagina) and first estrus (first day with cornified cells determined by  
131 daily [morning] vaginal cytology) during 45 days after the day of VO. In addition, estrous cyclicity  
132 was monitored by daily vaginal cytology, for a period of 30 days, in young (3 months old) and  
133 older (8 months old) WT and *Tac1/Tac2* KO (n≥8). Cytology samples were obtained every  
134 morning (10 am) and placed on a glass slide for determination of the estrous cycle under the  
135 microscope as previously described<sup>24</sup>.

136

137 **Study 2: Fecundity test in *Tac1/Tac2* KO male and female mice.**

138 In this study, adult WT (n=3) or *Tac1/Tac2* KO (n=7) male littermate mice (>75d) were placed with  
139 proven fertile WT females and time to delivery and number of pups per litter were monitored. In  
140 females, the fertility assessment was performed by breeding adult WT (n=3) or *Tac1/Tac2* KO  
141 (n=10) females with WT males previously proven to father litters. The time to first litter and number  
142 of pups per litter were recorded.

143 Additionally, the testes' ultra-structure was analyzed in adult (3–4 month old) mice of the two  
144 genotypes: WT and *Tac1/Tac2* KO (n=4/group). Testes were collected, weighed and fixed in  
145 Bouin's solution. The tissues were embedded in paraffin and sectioned (10 μm) for hematoxylin  
146 and eosin staining (Harvard Medical School Rodent Pathology Core) and images acquired under  
147 ×4 magnification. In females, the ovarian ultra-structure was also analyzed in adult (3–4 month  
148 old) mice of the two genotypes: WT and *Tac1/Tac2* KO (n=4/group). Ovaries were collected and  
149 processed as described above for the testes. The ovaries were analyzed for presence of corpora  
150 lutea (CLs) per section. Each value represents the number of CLs of 1 representative section  
151 from the middle line of one ovary per animal.

152

153 **Study 3: Characterization of the postgonadectomy response of LH in male and female mice.**



154 Bilateral removal of testes from 3-4-month-old males was performed with light isoflurane  
155 anesthesia. Briefly, the ventral skin was shaved and cleaned to perform one small incision in the  
156 skin and abdominal musculature. Once the gonads were identified and excised, the muscle  
157 incision was sutured, and the skin was closed with surgical clips. LH levels were measured in WT  
158 (n=13) and *Tac1/Tac2* KO (n=10) mice. Blood samples were collected before and then 2d and 7d  
159 after bilateral gonadectomy (GNX). Adult female mice were subjected to bilateral ovariectomy  
160 (OVX) via abdominal incision under light isoflurane anesthesia and LH levels were measured in  
161 intact (diestrus in the morning) WT (n=9) and *Tac1/Tac2* KO (n=6) adult (3-4 month) females and  
162 compared with 2d and 7d (days) after OVX.

163

164 **Study 4:** *Characterization of the LH pulsatile secretion in males and females.*

165 We assessed the pulsatile secretion of LH in adult GNX male (4-week after GNX) *Tac1/Tac2* KO  
166 mice and WT littermates (n=3-4 per group). Mice were handled daily to allow acclimation to  
167 sampling conditions for three weeks prior to the experiment. Pulsatile measurements of LH  
168 secretion were assessed by repeated blood collection through a single excision at the tip of the  
169 tail, as described previously<sup>25</sup>. The tail was cleaned with saline and then massaged to take a 4  
170 ul blood sample with a pipette. Whole blood was immediately diluted in 116 ul of 0.05% PBST,  
171 vortexed, and frozen on dry ice. Samples were stored at -80°C for a subsequent LH ELISA. We  
172 collected sequential blood samples every 10 min over a 150 minutes sampling period. Also, we  
173 assessed the pulsatile secretion of LH in adult OVX female (4 weeks after OVX) *Tac1/Tac2* KO  
174 mice and WT littermates (n=6-7 per group), following the protocol described above for males.

175

176 **Study 5:** *Expression of *Tacr1*, *Tacr2*, *Tacr3*, *Kiss1* and *Pdyn* in the mediobasal hypothalamus*  
177 *(MBH) of female mice.*

178 We aimed to determine if there are changes in the expression of *Tacr1*, *Tacr2*, *Tacr3*, *Kiss1*, and  
179 *Pdyn* in the mediobasal hypothalamus (MBH), the site that includes the arcuate nucleus (ARC)

180 between WT and *Tac1/Tac2* KO intact females. The hypothalami were dissected taking as limits  
 181 the posterior margin of the optic chiasm (rostrally) and the anterior margin of the mammillary  
 182 bodies (caudally), with a dissection depth of approximately 2 mm. Each hypothalamic sample was  
 183 dissected and divided into two, the suprachiasmatic region (the preoptic area, POA) or the MBH  
 184 and fragments were stored at  $-80^{\circ}\text{C}$  until further processing.

185 Total RNA from the MBH was isolated using TRIzol reagent (Invitrogen) followed by  
 186 chloroform/isopropanol extraction. RNA was quantified using NanoDrop 2000 spectrophotometer  
 187 (Thermo Scientific), and 1  $\mu\text{m}$  of RNA was reverse transcribed using iScript cDNA synthesis kit  
 188 (Bio-Rad). Quantitative real-time PCR assays were performed on an ABI Prism 7000 sequence  
 189 detection system, and analyzed using ABI Prism 7000 SDS software (Applied Biosystems). The  
 190 cycling conditions were the following: 2 min incubation at  $95^{\circ}\text{C}$  (hot start), 45 amplification cycles  
 191 ( $95^{\circ}\text{C}$  for 30 s,  $60^{\circ}\text{C}$  for 30 s, and 45 s at  $75^{\circ}\text{C}$ , with fluorescence detection at the end of each  
 192 cycle), followed by melting curve of the amplified products obtained by ramped increase of the  
 193 temperature from 55 to  $95^{\circ}\text{C}$  to confirm the presence of single amplification product per reaction.  
 194 For data analysis, relative standard curves were constructed from serial dilutions of one reference  
 195 sample cDNA and the input value of the target gene was standardized to *Hprt* levels in each  
 196 sample. The primers used are listed below.

Gene Name	Primer Sequence	Gene Accession Number
<i>Hprt</i>	F: CCTGCTGGATTACATTAAGCGCTG R: GTCAAGGGCATATCCAACAACAAAC	NM_013556.2
<i>Tacr1</i>	F: GTCTGCCAAGAGCCAAGAAC R: CCAGCCACATCTGAGAGACA	NM_009313
<i>Tacr2</i>	F: TCAACTTCATCTATGCCAGTCAC R: ATGACAGCAATAACCGCCTTG	NM_009314

<i>Tacr3</i>	F: GCCATTGCAGTGGACAGGTAT R: ACGGCCTGGCATGACTTTTA	NM_021382.6
<i>Kiss1</i>	F: CTCTGTGTCGCCACCTATGC R: TTCCCAGGCATTAACGAGTTC	AF472576.1
<i>Pdyn</i>	F: ACAGGGGGGAGACTCTCATCT R: GGGGATGAATGACCTGCTTACT	NM_018863.4

197 Abbreviations: F, forward; R, reverse.

198

199 **Study 6:** *Characterization of the estradiol-induced luteinizing hormone surge.*

200 In this study, WT (n=5), *Tac1* KO (n=5), *Tac2* KO (n=5) and *Tac1/Tac2* KO (n=5) adult female  
201 mice were subjected to bilateral OVX via abdominal incision under light isoflurane anesthesia.  
202 Immediately after OVX, capsules filled with E<sub>2</sub> (1ug/20g BW) were implanted subcutaneously (sc)  
203 via a small midscapular incision at the base of the neck; five days later, mice were subcutaneously  
204 injected in the morning with estradiol benzoate (1ug/20g BW) to produce elevated proestrus-like  
205 E<sub>2</sub> levels (LH surge) on the following day. Blood samples were collected at 10:00h and 19:00-  
206 19:30h<sup>26</sup>; LH levels were measured via ELISA.

207

208 **Study 7:** *Effect of NK1R and NK3R antagonists in the estradiol-induced luteinizing hormone*  
209 *surge in female mice.*

210 In this study, we aimed to evaluate whether substance P and NKB signaling is required to induce  
211 the preovulatory LH surge in WT females (n=5/group). An NK1R-antagonist (5 mg/kg), NK3R-  
212 antagonist (5 mg/kg) or vehicle (5% DMSO) were administered during the morning (10:00h) and  
213 afternoon (17:00h) of the day of the LH surge. The LH surge was induced following the protocol  
214 described above. Blood samples were collected at 10:00h (before the first injection of the  
215 antagonist) and 19:00-19:30h and LH levels were measured via ELISA.

216

217 **Hormone measurements:** LH was measured by a sensitive sandwich ELISA for the assessment  
218 of whole blood LH concentrations previously described elsewhere<sup>25</sup>. A 96-well high-affinity binding  
219 microplate (9018; Corning) was coated with 50  $\mu$ L of capture antibody (monoclonal antibody, anti-  
220 bovine LH beta subunit, 518B7; University of California) at a final dilution of 1:1000 (in 1XPBS,  
221 1.09 g of Na<sub>2</sub>HPO<sub>4</sub> [anhydrous], 0.32 g of NaH<sub>2</sub>PO<sub>4</sub> [anhydrous], and 9g of NaCl in 1000 mL of  
222 distilled water) and incubated overnight at 4°C. To minimize unspecific binding of the capture  
223 antibody, wells were incubated with 200  $\mu$ L of blocking buffer (5% [w/v] skim milk powder in  
224 1XPBS-T (1XPBS with 0.05% Tween20) for 2 hours at room temperature (RT). A standard curve  
225 was generated using a 2-fold serial dilution of LH (reference preparation, AFP-5306A; National  
226 Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Pituitary Program  
227 [NIDDK-NHPP]) in 0.2% (w/v) BSA-1XPBS-T. The LH standards and blood samples were  
228 incubated with 50  $\mu$ L of detection antibody (polyclonal antibody, rabbit LH antiserum,  
229 AFP240580Rb; NIDDK-NHPP) at a final dilution of 1:10000 for 1.5 hours (at RT). Each well  
230 containing bound substrate was incubated with 50  $\mu$ L of horseradish peroxidase conjugated  
231 antibody (poly-clonal goat anti-rabbit, D048701-2; DakoCytomation) at a final dilution of 1:2000.  
232 After a 1.5-hour incubation, 100  $\mu$ L of o-phenylenediamine (002003; Invitrogen), substrate  
233 containing 0.1% H<sub>2</sub>O<sub>2</sub> was added to each well and left at RT for 30 minutes. The reaction was  
234 stopped by addition of 50  $\mu$ L of 3M HCl to each well, and absorbance of each well was read at a  
235 wave length of 490 nm (Sunrise; Tecan Group). The concentration of LH in whole blood samples  
236 was determined by interpolating the OD values of unknowns against a nonlinear regression of the  
237 LH standard curve<sup>25</sup>. The reported intra- and inter-assay coefficients of variation for this assay  
238 are 6.05% and 4.29%, respectively<sup>25</sup>.

239

240 **Statistical Analysis:** All data are expressed as the mean  $\pm$  SEM for each group. A two tailed  
241 unpaired t-Student test or a one- or two-way ANOVA test followed by Newman-Kuel's or repeated  
242 measures Fisher's *post-hoc* test was used to assess variation among experimental groups.

243 Significance level was set at  $P < 0.05$ . All analyses were performed with GraphPad Prism  
244 Software, Inc (San Diego, CA).

245

246 **Statistical Analysis of LH pulses:** Mice LH concentration time series were analyzed using a  
247 custom-made MATLAB-based algorithm. It is a for loop written in the code to determine which LH  
248 peaks are considered pulses. This for loop states that any value whose height is 20% greater  
249 than the heights of the 2 previous values as well as 10% greater than the height of the following  
250 value is considered a pulse. There is also a condition written into the code that is specific for the  
251 second time interval ( $i=2$ ) that states that the value at the second-time interval only needs to be  
252 20% greater than the single value that comes before it to be considered a pulse.

253

## 254 **RESULTS**

### 255 **Absence of tachykinin signaling delays puberty onset in males and females.**

256 As expected, *Tac1/Tac2KO* mice showed delayed puberty onset in males (assessed by the age  
257 of preputial separation) (**Figure 1A, B**) despite normal body weight (BW) (**Figure 1C**).  
258 Interestingly, although *Tac1/Tac2KO* female mice had normal timing of vaginal opening (VO),  
259 there was no evidence of first estrus (marker for central activation of the reproductive axis) during  
260 the time of the study (45 days after VO), despite similar BW to controls (**Figure 1D-G**). Next, in  
261 order to better assess the fitness of the reproductive axis in females, estrous cyclicity was  
262 monitored for 30 days at 3 months of age (3 mo) and 8 mo. These ages were selected based on  
263 previous studies in *Tac2KO* females, which showed absent or irregular estrous cycles during the  
264 early adult phase (3 mo) that progressed into regular cycles by the age of 8 mo<sup>17</sup>. *Tac1/Tac2KO*  
265 females mice, however, presented disrupted estrous cycles at both ages, spending most of their  
266 time in diestrus with some few sporadic estrous phases over a 30 day period (**Figure 1 H,I**).

267

### 268 **Tachykinin signaling is necessary for normal fertility in females, but not males.**

269 In order to determine whether the delay in sexual maturation observed in peripubertal  
270 *Tac1/Tac2*KO male and female mice affects fertility as suggested by the impaired estrous cycles  
271 (**Figure 1**), *Tac1/Tac2*KO mice (both sexes) were subjected to a fecundity test. KO mice were  
272 housed with proven fertile WT mates for 2,5 months. As a result, 100% of the males fathered  
273 litters that were normal in size (**Figure 2A**). The latency to impregnate females was also similar  
274 between groups (**Figure 2B**). In addition, males displayed normal testicular weight and histology  
275 with the presence of mature sperm (**Figure 2C,D**). Furthermore, in order to test the response of  
276 central elements of the HPG axis to the absence of negative feedback, males were castrated and  
277 LH samples collected before surgery (basal) and 2 and 7 days post-surgery. Interestingly, the  
278 compensatory rise of LH was present in WT, but not in KO mice, at 2d post-castration, however,  
279 the LH level in KOs reached the level of the control group by 7d (**Figure 2E**).

280 On the other hand, the majority (80%) of the female KOs were infertile. In the remaining 20% of  
281 the females that got pregnant, the litter size was significantly smaller and pregnancy latency  
282 significantly longer than in controls (**Figure 2F-H**). This was accompanied by smaller ovarian size,  
283 and significantly fewer corpora lutea (CL), suggesting an ovulation impairment (**Figure 2I-K**)  
284 despite a slightly higher body weight than controls ( $W = 21.75\text{g} \pm 1.06$ ; *Tac1/Tac2*KO =  $25.0\text{g} \pm 0.76$ ; \* $p < 0.05$ ). However, at least one CL could be found in 75% of the ovaries assessed in  
285 *Tac1/Tac2*KO females. As in males, the response to the removal of the negative feedback after  
286 ovariectomy was lower at 2d post-surgery, however, in these females, the LH level did not reach  
287 statistical significance compared to controls at 2d post-surgery. Nonetheless, LH levels did not  
288 increase from basal to 2d post OVX, resembling the effect in male KOs, while a significant LH rise  
289 2d post-surgery was already detected in control animals. LH levels in KO mice recovered to  
290 control levels by 7d (**Figure 2L**).

292

293 **Absence of tachykinin signaling decreases frequency and amplitude of LH pulses.**

294 The pattern of pulsatile LH release was assessed every 10 min for 150 min in WT and  
295 *Tac1/Tac2*KO mice of both sexes in a model of elevated LH pulsatility, i.e. four weeks after  
296 gonadectomy. Thus, *Tac1/Tac2*KO mice showed decreased frequency of LH pulses (that reached  
297 statistical significance in females) and decreased amplitude in males determined by the AUC of  
298 the total secretory LH mass (**Figure 3A-F**).

299 Despite significant alteration in the overall pattern of LH release, the expression of the genes  
300 involved in the shaping of kisspeptin pulses: *Kiss1*, *Pdyn* and the tachykinin receptors *Tacr1*,  
301 *Tacr2* and *Tac3r* was similar between both groups in the MBH (Suppl Figure 1).

302

### 303 **Tachykinin signaling is necessary for the preovulatory LH surge.**

304 The lower number of CL(s) in females suggested that the infertile phenotype of 80% of the  
305 *Tac1/Tac2*KO females was due to an ovulatory deficit. In order to evaluate the role of tachykinins  
306 on the positive feedback of sex steroids required for ovulation, WT, *Tac1*KO, *Tac2*KO and  
307 *Tac1/Tac2*KO female mice were subjected to an LH surge-inducing protocol and blood was  
308 collected in the morning (~10:00h) and evening, after lights off (~19:00h). KO mice for a single  
309 *Tac* gene (i.e. *Tac1* or *Tac2*) displayed a discernible LH surge, that was not statistically different  
310 from controls. However, *Tac1/Tac2*KO mice failed to display an LH surge (**Figure 4A**). This finding  
311 suggests that the single *Tac* KO models develop a compensatory mechanism, likely driven by the  
312 remaining (intact) tachykinin system. In order to test whether this compensation is acquired  
313 throughout development in the chronic absence of a tachykinin system or it is already present in  
314 WT mice, we subjected a new cohort of WT females to the same LH surge protocol used in KO  
315 mice. In this case, mice were also injected i.p. with specific antagonists of NK1R or NK3R in the  
316 morning (10:00h) and afternoon (17:00h). Both antagonists individually were able to block the LH  
317 surge (**Figure 4B**).

318

### 319 **DISCUSION**

320 The role of tachykinins in regulating reproductive function has been described in several  
321 species. Tachykinins (NKA, NKB and SP) have been shown to significantly stimulate  
322 gonadotropin release (in a sex steroid-dependent manner in the case of NKA and NKB)<sup>1,27</sup>. The  
323 present study extends our knowledge of the role of tachykinins in puberty onset, the generation  
324 of GnRH pulses and mounting of the preovulatory LH surge.

325

326 Tachykinins are required for the proper timing of puberty onset:

327 Overall, the action of tachykinins on the HPG axis has been documented from early  
328 developmental stages, where NKB has been posed with the critical role of serving as a stimulator  
329 of kisspeptin release to awaken the reproductive axis, therefore, kickstarting puberty onset<sup>1,27,28</sup>.  
330 Studies using single deficiency of tachykinin models (*Tac1*KO and *Tac2*KO) have exhibited a  
331 delay in puberty onset in males and females, supporting that not only NKB, but also NKA/SP have  
332 a role in the timing of the pubertal release of kisspeptin/GnRH. However, all *Tac1*KO and *Tac2*KO  
333 mice eventually displayed signs of sexual maturation (PS in males and VO + first estrus in  
334 females)<sup>11,17-19</sup>. Of note, these studies describing the phenotype of the single KO models were  
335 performed in our facility under the same conditions as the present studies in *Tac1/Tac2*KO mice.  
336 Interestingly, while *Tac1/Tac2*KO male mice resembled the phenotype of *Tac1*KO mice (i.e. 4-5  
337 day delay in PS)<sup>19</sup>, female *Tac1/Tac2*KO mice presented normal time of VO but absence of first  
338 estrus, unlike the single KO models<sup>11,17</sup>. The reason for this discrepancy in the timing of VO is  
339 unclear and suggests the existence of sufficient circulating levels of estradiol (E2) to induce the  
340 normal progression of this pubertal marker. Nonetheless, while VO is a direct measure of  
341 circulating E2 levels, the absence of detectable first estrus for 45 days following VO in the double  
342 KO females denotes a central impairment in the normal ovulatory process. This impairment is  
343 more severe than in single KO mouse models, which display detectable first estrus shortly after  
344 VO<sup>11,17</sup>.



345 Furthermore, the impairment to achieve estrus in *Tac1/Tac2KO* female mice is maintained  
346 throughout the life of the mouse (at 3 and 8 mo) with only sporadic estrus observed in some  
347 females (<2 estrous phases in 30 days). This phenotype is in direct contrast to that of *Tac1KO*  
348 females, which display regular estrous cycles<sup>11</sup>; and *Tac2KO* females, which transition from  
349 irregular cycles at 3 mo to regular ones at 8 mo<sup>17</sup>.

350

351 Tachykinins are essential for the generation of the preovulatory LH surge:

352 The sporadic estrus observed in some *Tac1/Tac2KO* females is in line with the 20% of  
353 pregnancies observed in these mice, in striking contrast to the 100% success rate of pregnancies  
354 in the single KO models. Of note, despite their fertile phenotype, single *Tac1* and *Tac2* KO models  
355 present smaller ovaries and fewer CL that led to smaller litters in *Tac1KO*, but not in *Tac2KO*<sup>11,17</sup>.  
356 However, in the fraction of *Tac1/Tac2KO* mice that got pregnant, the litter size was even smaller  
357 than in *Tac1KO* females and the pregnancy latency was larger than in both single KO models,  
358 highlighting the more severe reproductive impairment in the complete absence of tachykinin  
359 signaling. This phenotype in *Tac1/Tac2KO* female mice strongly suggested a failure to mount a  
360 proper preovulatory LH surge. Because ovulation has not been studied in previous experiments  
361 characterizing the single tachykinin KO models, we set out to compare the ability of each model  
362 to display an LH surge compared to WT littermate controls. Interestingly, *Tac1KO* and *Tac2KO*  
363 mice presented a trend to smaller increases in LH release (LH surge) than controls. This effect is  
364 in line with the lower number of CL(s) described for each model although the amount of LH  
365 released is sufficient to attain successful pregnancies in all of the animals of each group<sup>11,17</sup>. Not  
366 surprisingly, given the severe decrease in the fertility rate among *Tac1/Tac2KO* females, we did  
367 not observe an LH surge in any of the animals tested. This defect suggests that the products of  
368 both tachykinin genes are required for full ovulatory capabilities in females. The absence of one  
369 of the tachykinin systems reduces the magnitude of the LH surge, in accordance with the  
370 decreased fertility rate of single KO females<sup>11,17</sup>; however, the complete blunting of the LH surge

371 in the absence of both tachykinin systems leads to complete infertility in 80% of the females. This  
372 is in line with the reversal phenotype of the hypogonadal state described in patients with NKB  
373 signaling deficiency, which has been speculated to be mediated by NKA/SP. Nonetheless,  
374 NKA/SP deficiency in patients (*TAC1/TACR1/TACR2* mutants) has not been associated with  
375 hypogonadism or fertility impairments, despite the documented stimulatory effect of SP on LH  
376 release in humans<sup>29</sup>. Thus, we can infer that the absence of *TAC1* (SP/NKA) signaling in humans  
377 induces a milder effect on fertility than the absence of *TAC3* (NKB), resembling the fertile  
378 phenotype of *Tac1* deficient mice.

379           Nonetheless, the decrease in the fertility rate of double KO females contrasts with the fact  
380 that 75% of the ovaries of these females showed at least one CL. While this explains the reduction  
381 in the number of pups per litter, it does not explain the 80% infertility rate. To interpret these data,  
382 it is important to put in context the potential extent of the contribution of the tachykinin systems to  
383 reproduction, which might involve a role in sexual behavior. We have recently identified a  
384 population of NK3R expressing neurons in the medial amygdala (MeA) of the mouse that can  
385 significantly stimulate LH release<sup>30</sup>. Because the amygdala is critical for sexual behavior<sup>31-34</sup>, it is  
386 therefore plausible that *Tac1/Tac2*KO females present a behavioral deficit, such as lordosis  
387 impairments, that further contributes to the infertile phenotype despite the presence of sporadic  
388 ovulation (i.e. CLs).

389           Of note, the existence of CL in mice with reduced magnitude of their preovulatory LH surge  
390 is consistent with previous reports in mice<sup>35</sup>, which suggests that the required magnitude of an  
391 LH surge to induce ovulation is substantially lower than what is normally achieved in a WT mouse.  
392 This lower requirement of the magnitude of the LH surge might account for the sporadic ovulation  
393 observed in some *Tac1/Tac2*KO females.

394           We further investigated this novel role of tachykinins in the induction of the LH surge by  
395 assessing the effect of acutely blocking the NKB or SP receptor (NK3R and NK1R, respectively)  
396 in adult WT females subjected to a similar LH surge inducing protocol as was previously carried

397 out in the KO models. Surprisingly, both antagonists alone were able to suppress the LH surge  
398 when injected in the morning and afternoon of the day of the expected LH surge, further  
399 highlighting that both tachykinin systems have an active role in triggering ovulation. This effect is  
400 in line with previous reports in humans and monkeys indicating that NK1R and NK3R antagonists  
401 delay the LH surge<sup>36,37</sup>. Moreover, the NK3R agonist senktide induces an LH surge in ewes during  
402 the follicular phase when injected into the retrochiasmatic and preoptic areas<sup>38,39</sup>. Our current  
403 data in mice document a previously unknown but critical role of tachykinins in ovulation and,  
404 furthermore, demonstrate that compensation of the congenital absence of one of these tachykinin  
405 systems can develop in mice, likely as an evolutionary fail-safe mechanism to maintain  
406 reproductive function.

407 Furthermore, the lack of LH surge after acute blockade of one of the tachykinin receptors  
408 suggests that there is no duplication in their role that could lead to an additive effect – as could  
409 be inferred by the decrease in the magnitude of the LH surge by approximately half of the normal  
410 magnitude observed in each individual KO model. This experiment supports the contention that  
411 there is, indeed, an acquired capacity to ovulate in congenitally deficient single tachykinin deficient  
412 models. Whether this compensation is achieved by the additional tachykinin system taking over,  
413 for example SP/NK1R in the absence of NKB signaling, or through the cross activation of the  
414 different ligand/receptors, such as SP acting on NK3R, is yet unknown. In any case, this effect  
415 might occur directly at the level of Kiss1 neurons in the AVPV/PeN (Kiss1<sup>AVPV/PeN</sup>, which are  
416 responsible for mounting the LH surge by releasing a surge of kisspeptin in response to rising E2  
417 levels) and/or GnRH neurons since both populations of neurons express NK1R and NK3R in  
418 similar percentages (25% and 10%, respectively)<sup>6</sup>.

419

#### 420 GnRH pulses can occur in the absence of tachykinin signaling:

421 In addition to the novel role in the induction of the LH surge discussed above, tachykinins  
422 have been posed as components of the GnRH pulse generator, specially the NKB/NK3R system,

423 by stimulating the pulsatile release of kisspeptin from Kiss1<sup>ARC</sup> neurons. However, we have  
424 recently demonstrated in mice that in the absence of NKB signaling (*Tac2KO*), LH pulses are still  
425 present albeit at a slower frequency and smaller amplitude, in line with NKB signaling deficient  
426 patients<sup>40,41</sup>. Importantly, this alteration in LH pulsatility did not affect fertility in mice and was  
427 sufficient to reverse infertility in human mutants, suggesting that successful reproduction can be  
428 achieved with a minimal baseline of LH pulses.

429 Interestingly, the concept of the role of NKB (in coordination with dynorphin) as the source  
430 of the LH pulses is challenged by studies showing that continuous kisspeptin infusion in NKB  
431 signaling deficient patients, as well as in a sheep model of pharmacological antagonism of  
432 NK3R<sup>42</sup>, leads to an increase in LH pulsatility – suggesting that Kiss1<sup>ARC</sup> neurons are not the  
433 GnRH pulse generator or that additional pulse generators exist or develop. However, this effect  
434 (i.e. induction of LH pulses after kisspeptin administration) is not observed in *Tac2KO* mice, which  
435 only respond with a sustained increase in circulating LH levels after kisspeptin treatment<sup>40</sup>. While  
436 species differences might exist, important questions remain to fully reconcile these data in  
437 different species and thus understand the whole mechanism underlying GnRH pulses. For  
438 instance, the data in humans and sheep suggest the existence of a pulsatile inhibitory factor that  
439 must act on GnRH neurons to cease every pulse of GnRH after kisspeptin stimulation. Otherwise,  
440 each pulse of kisspeptin would depolarize GnRH neurons for up to 30 min<sup>43</sup> (or longer after  
441 continuous kisspeptin administration), while it is known that LH pulses occur as frequently as  
442 every < 10 min<sup>44</sup> in gonadectomized mice. This pulsatile inhibition upon GnRH neurons might be  
443 mediated by dynorphin based on studies in sheep<sup>45</sup>.

444 Initial hypotheses for the presence of rudimentary LH pulses in NKB deficient mice and  
445 humans included compensation by NKA/SP. Our current data in complete tachykinin deficient  
446 mice (i.e. *Tac1/Tac2KO* mice) debunks this hypothesis. We demonstrate that LH pulsatility is  
447 preserved in the complete absence of tachykinins, albeit at a lower frequency, resembling the  
448 profile of *Tac2KO* mice<sup>40</sup>. This phenotype indicates that the source of these rudimentary LH pulses

449 is not the compensation by other tachykinins. As in *Tac2*KO mice, basal LH pulses in  
450 *Tac1/Tac2*KO mice are able to maintain a tonic release of LH that is sufficient to stimulate the  
451 synthesis and release of sex steroids to achieve sexual maturation (PS and VO, although with a  
452 certain delay compared with controls). These basal LH pulses are therefore enough to activate  
453 the HPG axis, as evidenced by the normal fertility and testicular histology in *Tac1/Tac2*KO males.  
454 In females, this low stimulation of the ovary might be sufficient to induce gametogenesis and some  
455 estradiol production that might be insufficient for normal gonadal development, leading to smaller  
456 ovaries and large periods in diestrus, in addition to the ovulatory impairments described above.

457         Nonetheless, the presence of circulating sex steroids in male and female double KO mice  
458 is further evidenced by the absence of changes in the expression of the genes encoding the  
459 known ligands and the receptors controlling kisspeptin release in Kiss1<sup>ARC</sup> neurons (*Kiss1*, *Pdyn*,  
460 *Tacr1* and *Tacr3*). The expression of these genes is highly regulated (inhibited) by circulating sex  
461 steroids<sup>5,6</sup>. Therefore, the lack of change observed in the expression of these genes indicates that  
462 there is a sufficient sex steroid level circulating in the animal to hold their expression down to  
463 control levels.

464         Moreover, this study in *Tac1/Tac2*KO uncovered an important sex difference in the  
465 mechanism/s underlying the generation of GnRH/LH pulses as observed by the significantly lower  
466 amplitude of LH pulses in male (but not female) gonadectomized *Tac1/Tac2*KO mice compared  
467 to controls. This difference might indicate the existence of sexual dimorphism in the mechanisms  
468 underlying GnRH pulses that remains to be further characterized.

469         The main question arising from the present data is, *what drives these rudimentary LH*  
470 *pulses?* These pulses could be explained by the existence of yet another pulse generator  
471 upstream of Kiss1<sup>ARC</sup> neurons or, a more likely explanation, the pace-maker activity of Kiss1<sup>ARC</sup>  
472 neurons, which has been already demonstrated<sup>46</sup>. In this context, tachykinins would act as  
473 modulators of the activity of the pace-maker. Along these lines, our data demonstrated a delay in  
474 the LH response to the removal of the negative feedback of sex steroids in both sexes. While LH

475 was significantly elevated 2 days after gonadectomy in WT mice, LH levels in KOs of both sexes  
476 remained unchanged at this time (with LH levels reaching that of WTs by 7d post-surgery). This  
477 delay suggests a role for tachykinins as accelerators of kisspeptin pulsatility to adapt to changing  
478 levels of sex steroids. Then, upon removal of sex steroids, tachykinins in WT mice would rapidly  
479 increase the frequency and amplitude of kisspeptin pulses in an attempt to achieve a rapid return  
480 to normal sex steroid levels (**Figure 5**). The same mechanism would apply in the regulation of the  
481 timing of puberty onset. Thus, in the absence of tachykinins (i.e. the accelerator of the  
482 endogenous pace-maker activity), puberty onset would take longer, as we have observed in the  
483 tachykinin deficient models. This action of tachykinins might occur directly on Kiss1<sup>ARC</sup> neurons,  
484 which express NK1R and NK3R (50% and >90%, respectively)<sup>6</sup> and have been shown to directly  
485 respond to SP and NKA in addition to NKB<sup>47</sup>.

486

487 Overall, our findings confirmed a role for tachykinins in the proper timing of puberty onset  
488 and fine-tuning of pulsatile LH release to circulating sex steroids. However, these data evidence  
489 that tachykinins are not necessary for LH pulses, which remain present at a lower, basal, rate in  
490 their absence, being sufficient to achieve puberty onset and fertility in male mice. Finally, we show  
491 for the first time that NK1R and NK3R signaling are necessary for the formation of the preovulatory  
492 LH surge in females. However, in the congenital absence of one of these systems, compensation  
493 from the other one takes place in order to preserve fertility. This role of tachykinins in ovulation  
494 raises novel possibilities for the use of agonists and antagonists of tachykinin receptors in fertility  
495 induction protocols and as novel non-steroidal contraceptive approaches.

496

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629 **Figure Legends:**

630 **Figure 1: Pubertal progression in male and female *Tac1/Tac2KO* mice.** (A,B) Pubertal  
631 progression in males documented by preputial separation (PS) and body weight (BW) at the time  
632 of PS (C) (n ≥ 11/group). (D,E) Pubertal progression in females documented by vaginal opening  
633 (VO), BW at VO (F) and first estrus (G) (n ≥ 8/group). Estrous cyclicity during 30 days at age 3  
634 months (H) and 8 months (I) (n ≥ 8/group). \*p<0.05, \*\*p<0.01. Student t test and One Way ANOVA  
635 followed by Newman Keuls *post hoc* test (H,I).

636

637 **Figure 2: Fecundity test, gonadal histology and response to gonadectomy in male and**  
638 **female *Tac1/Tac2KO* mice.** Litter size (A), parturition latency (B), testicular histology (C) and  
639 testicular weights (D) in WT and *Tac1/Tac2KO* males (n = 7/group). Circulating LH levels before  
640 (basal) and 2 and 7 days after castration (E) (n = 10/group). In females, percentage of fertile  
641 females during 11 weeks of mating (F), litter size (G), parturition latency (H), ovarian histology (I),  
642 number of corpora lutea (CL) (J) and ovarian weights (K) in WT vs *Tac1/Tac2KO* females (n =  
643 10/group). Circulating LH levels before (basal) and 2 and 7 days after ovariectomy (L). p<0.05,  
644 \*\*p<0.01, \*\*\* p<0.001 Student t test. Different letters indicate significantly different values after a  
645 Two-Way ANOVA and repeated measures Fisher's *post hoc* test.

646

647 **Figure 3: Pattern of LH pulsatility in gonadectomized WT and *Tac1/Tac2KO* mice.** LH  
648 samples were collected every 10 min for 150 min in males and females 4 weeks after  
649 gonadectomy. Pattern of LH pulses, number of pulses/150 min and total secretory mass assessed  
650 by area under the curve (AUC) in males (n ≥ 3/group) (A-C) and females (n ≥ 6/group) (D-F).  
651 \*\*p<0.01, Student t test. \* denotes an LH pulses following the protocol described in the methods.

652

653 **Figure 4: Preovulatory LH surge in *Tac1*KO, *Tac2*KO and *Tac1/Tac2*KO, and WT after NK1R**  
654 **and NK3R blockade.** Circulating LH measurements in the morning (10 am) and afternoon (7 pm)  
655 after an LH surge inducing protocol in WT, *Tac1*KO, *Tac2*KO and *Tac1/Tac2*KO mice (A) (n =  
656 5/group), and in WT after the administration of NK1R or NK3R antagonists (B) (n = 5/group).  
657 Different letters indicate significantly different values after a repeated measures Two-Way ANOVA  
658 and Fisher's *post hoc* test.

659

660 **Figure 5: Schematic representation of the pattern of pulsatile and surge LH release.** Kiss1  
661 neurons present intrinsic activity that leads to a basal level of tonic release of LH that is sufficient  
662 to maintain reproduction in males. In the presence of tachykinins, LH pulses acquire normal  
663 frequency and amplitude (red). In females, tachykinins are required for the formation of the  
664 preovulatory LH surge (red). Whether the action of SP/NKA and NKB is direct on Kiss1<sup>AVPV/PeN</sup>  
665 neurons or through intermediate neurons, as well as the neuronal population that is the source of  
666 these tachykinins, remain to be deciphered.

667

668 **Supplemental Figure 1: Expression of *Tacr1*, *Tacr2*, *Tacr3*, *Kiss1* and *Pdyn* in the**  
669 **mediobasal hypothalamus (MBH) of female mice.** Two-Way ANOVA and Newman Keuls *post*  
670 *hoc* test (n = 5/group).

671

672

## 673 **Statements**

### 674 **Statement of Ethics**

675 All animal studies were approved by the Brigham and Women's Hospital Institutional Animal  
676 Care and Use Committee.

677

678 **Disclosure Statement**

679 The authors have nothing to disclose.

680

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686

687 **Author Contributions**

688 SL, VMN designed the experiments; SL, CF, RT, CAM, AG performed the experiments; SBS  
689 provided essential material; SL, CF, VMN, discussed the data and wrote the manuscript.

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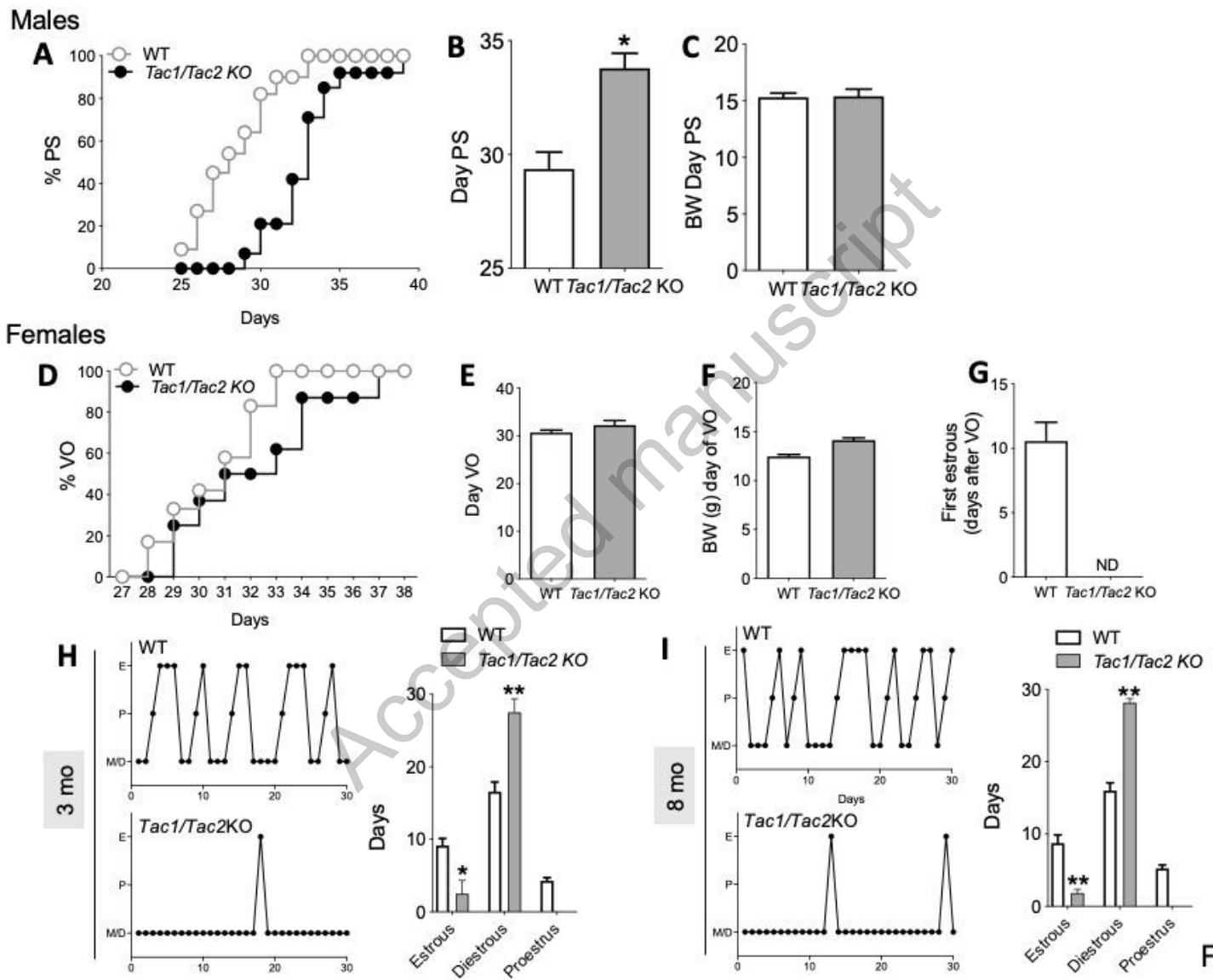


Figure 1

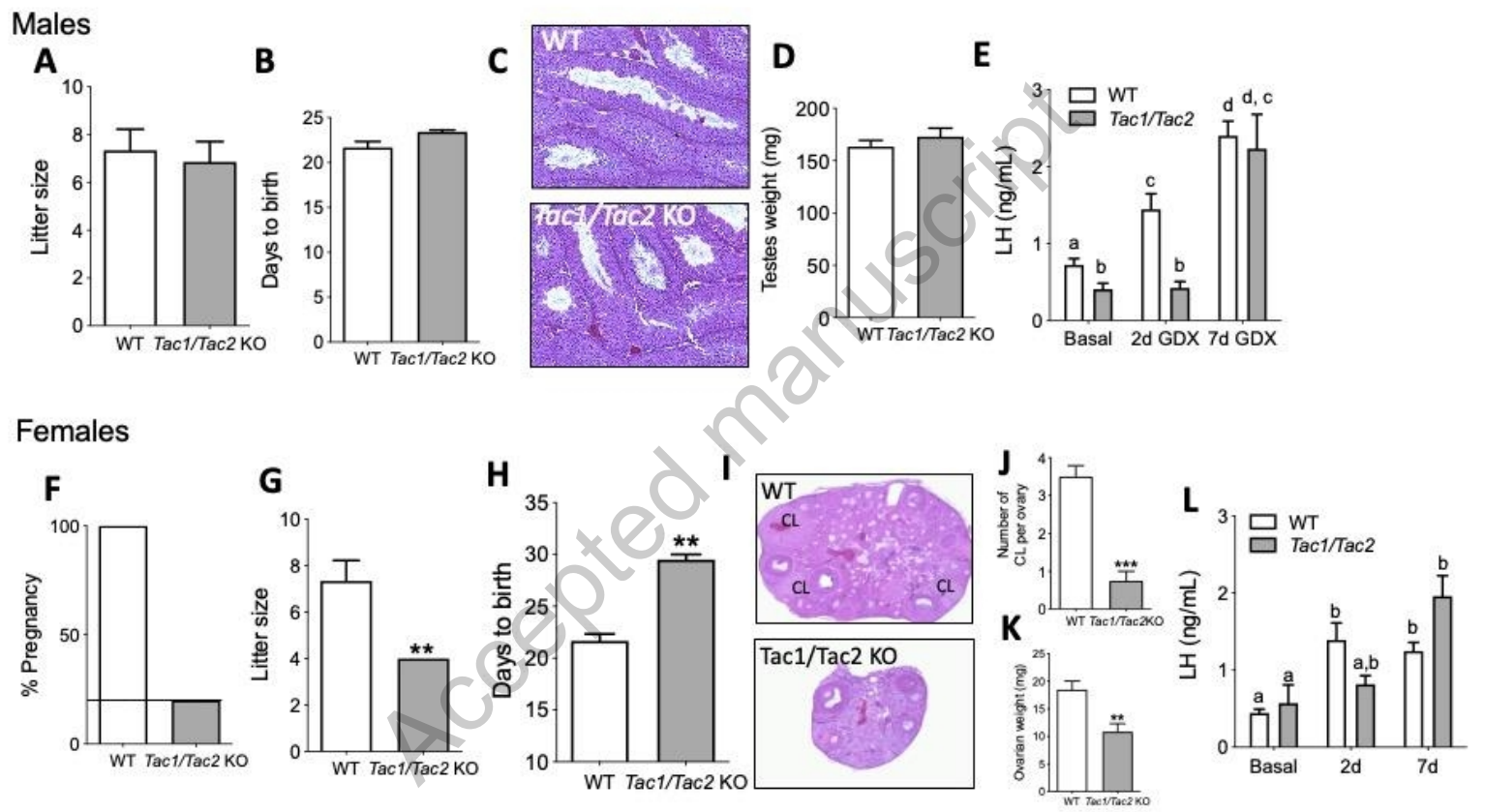
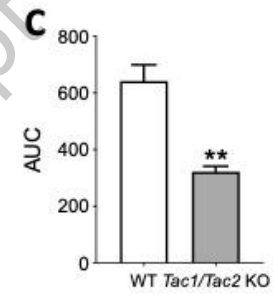
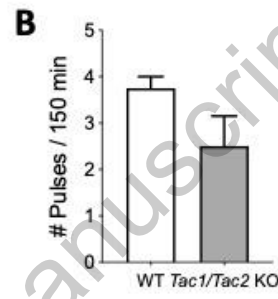
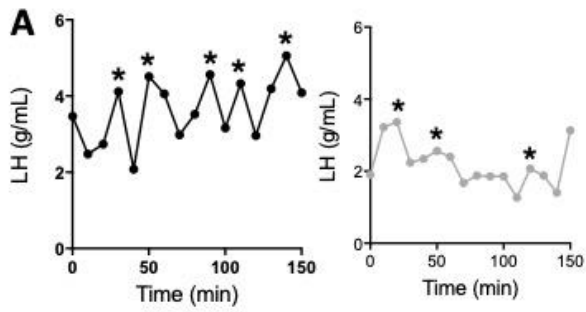


Figure 2

Males



Females

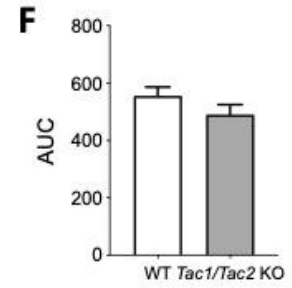
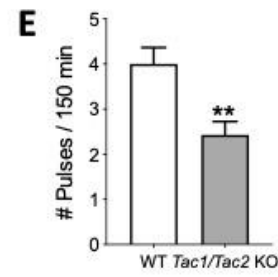
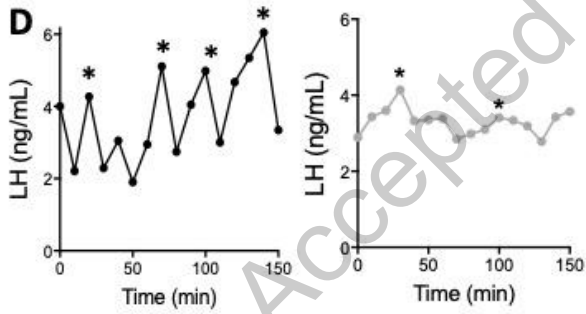


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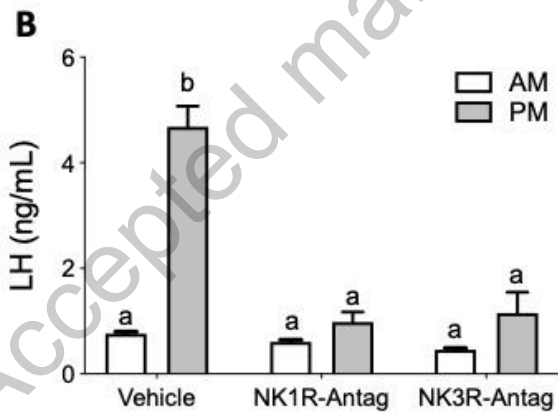
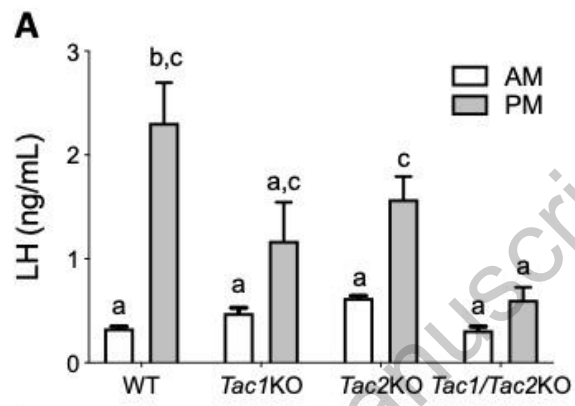
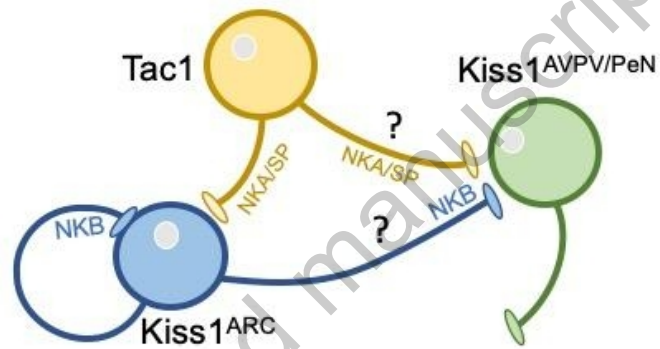


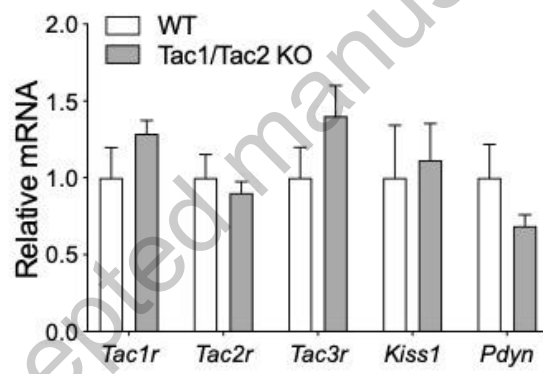
Figure 4





	Kiss1 <sup>ARC</sup>		Kiss1 <sup>AVPV/PeN</sup>	
	- E2/T	+ E2/T	- E2	+ E2/P
<b>Endogenous Kiss1 activity</b>				
<b>+ Tachykinins</b>				

Figure 5



Supplemental Figure 1