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

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ABSTRACT

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

INTRODUCTION

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

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

MATERIALS AND METHODS

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

Reagents: The antagonists of NK3R (SB 222200) and NK1R (RP67580) were purchased from Tocris Bioscience (Minneapolis, MN). Doses and timings for hormonal analyses were selected on the basis of previous studies[6,22,23].

Experimental design

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

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

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

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

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

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

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

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

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

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

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

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

between WT and *Tac1/Tac2* KO intact females. The hypothalami were dissected taking as limits the posterior margin of the optic chiasm (rostrally) and the anterior margin of the mammillary bodies (caudally), with a dissection depth of approximately 2 mm. Each hypothalamic sample was dissected and divided into two, the suprachiasmatic region (the preoptic area, POA) or the MBH and fragments were stored at −80 ºC until further processing.

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

Gene Name	**Primer Sequence**	**Gene Accession Number**
Hprt	F: CCTGCTGGATTACATTAAAGCGCTG R: GTCAAGGGCATATCCAACAACAAAC	NM_013556.2
Tacr1	F: GTCTGCCAAGAGCCAAGAAC R: CCAGCCACATCTGAGAGACA	NM_009313
Tacr2	F: TCAACTTCATCTATGCCAGTCAC R: ATGACAGCAATAACCGCCTTG	NM_009314

Tacr3	F: GCCATTGCAGTGGACAGGTAT R: ACGGCCTGGCATGACTTTTA	NM_021382.6
Kiss1	F: CTCTGTGTCGCCACCTATGC R: TTCCCAGGCATTAACGAGTTC	AF472576.1
Pdyn	F: ACAGGGGGAGACTCTCATCT R: GGGGATGAATGACCTGCTTACT	NM_018863.4

Abbreviations: F, forward; R, reverse.

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

In this study, WT (n=5), *Tac1* KO (n=5), *Tac2* KO (n=5) and *Tac1/Tac2* KO (n=5) adult female mice were subjected to bilateral OVX via abdominal incision under light isofluorane anesthesia. Immediately after OVX, capsules filled with E_2 (1ug/20g BW) were implanted subcutaneously (sc) via a small midscapular incision at the base of the neck; five days later, mice were subcutaneously injected in the morning with estradiol benzoate (1ug/20g BW) to produce elevated proestrus-like E_2 levels (LH surge) on the following day. Blood samples were collected at 10:00h and 19:00-19:30h [26]; LH levels were measured via ELISA.

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

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

Hormone measurements: LH was measured by a sensitive sandwich ELISA for the assessment of whole blood LH concentrations previously described elsewhere[25]. A 96-wellhigh-affinity binding microplate (9018; Corning) was coated with 50uL of capture antibody (monoclonal antibody, anti-bovine LH beta subunit, 518B7; University of California) at a final dilution of 1:1000 (in 1XPBS, 1.09 g of Na2HPO4 [an-hydrous], 0.32 g of NaH2PO4 [anhydrous], and 9g of NaCl in1000 mL of distilled water) and incubated overnight at 4°C. To minimize unspecific binding of the capture antibody, wells were incubated with 200uL of blocking buffer (5% [w/v] skim milk powder in 1XPBS-T (1XPBS with 0.05% Tween20) for 2hours at room temperature (RT). A standard curve was generated using a 2-fold serial dilution of LH (reference preparation, AFP-5306A; National Institute of Diabetes and Digestive and Kidney Diseases National Hormone and Pituitary Program [NIDDK-NHPP]) in 0.2% (w/v) BSA-1XPBS-T. The LH standards and blood samples were incubated with 50 uL of detection antibody (polyclonal antibody, rabbit LH antiserum, AFP240580Rb; NIDDK-NHPP) at a final dilution of 1:10000 for 1.5 hours (at RT). Each well containing bound substrate was incubated with 50 ul of horseradish peroxidase conjugated antibody (poly-clonalgoatanti-rabbit, D048701–2; DakoCytomation) at a final dilution of 1:2000. After a 1.5-hour incubation, 100Ul of o-phenylenediamine (002003;Invitrogen), substrate containing 0.1% H2O2 was added to each well and left at RT for 30minutes.The reaction was stopped by addition of 50 uL of 3M HCl to each well, and absorbance of each well was read at a wave length of 490 nm (Sunrise; Tecan Group). The concentration of LH in whole blood samples was determined by interpolating the OD values of unknowns against a nonlinear regression of the LH standard curve [25]. The reported intra- and inter-assay coefficients of variation for this assay are 6.05% and 4.29%, respectively[25].

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

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

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

RESULTS

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

As expected, *Tac1/Tac2*KO mice showed delayed puberty onset in males (assessed by the age of preputial separation) (**Figure 1A, B**) despite normal body weight (BW) (**Figure 1C**). Interestingly, although *Tac1/Tac2*KO female mice had normal timing of vaginal opening (VO), there was no evidence of first estrus (marker for central activation of the reproductive axis) during the time of the study (45 days after VO), despite similar BW to controls (**Figure 1D-G**). Next, in order to better assess the fitness of the reproductive axis in females, estrous cyclicity was monitored for 30 days at 3 months of age (3 mo) and 8 mo. These ages were selected based on previous studies in *Tac2*KO females, which showed absent or irregular estrous cycles during the early adult phase (3 mo) that progressed into regular cycles by the age of 8 mo[17]. *Tac1/Tac2*KO females mice, however, presented disrupted estrous cycles at both ages, spending most of their time in diestrus with some few sporadic estrous phases over a 30 day period (**Figure 1 H,I**).

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

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

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

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

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

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

Tachykinin signaling is necessary for the preovulatory LH surge.

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

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)[1,27]. 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[1,27,28].
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)[11,17-19]. 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)[19], female *Tac1/Tac2*KO mice presented normal time of VO but absence of first
338 estrus, unlike the single KO models[11,17]. 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[11,17].

345 Furthermore, the impairment to achieve estrus in *Tac1/Tac2*KO 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 *Tac1*KO
348 females, which display regular estrous cycles[11]; and *Tac2*KO females, which transition from
349 irregular cycles at 3 mo to regular ones at 8 mo[17].
350
351 Tachykinins are essential for the generation of the preovulatory LH surge:
352 The sporadic estrus observed in some *Tac1/Tac2*KO 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 *Tac1*KO, but not in *Tac2*KO[11,17].
356 However, in the fraction of *Tac1/Tac2*KO mice that got pregnant, the litter size was even smaller
357 than in *Tac1*KO 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/Tac2*KO 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, *Tac1*KO and *Tac2*KO
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[11,17]. Not
366 surprisingly, given the severe decrease in the fertility rate among *Tac1/Tac2*KO 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[11,17]; 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[29]. 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[30]. Because the amygdala is critical for sexual behavior[31-34], 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[35], 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

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

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

<u>GnRH pulses can occur in the absence of tachykinin signaling:</u>

In addition to the novel role in the induction of the LH surge discussed above, tachykinins 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^{ARC} neurons. However, we have
424 recently demonstrated in mice that in the absence of NKB signaling (*Tac2*KO), LH pulses are still
425 present albeit at a slower frequency and smaller amplitude, in line with NKB signaling deficient
426 patients[40,41]. 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[42], leads to an increase in LH pulsatility – suggesting that Kiss1^{ARC} 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 *Tac2*KO mice, which
435 only respond with a sustained increase in circulating LH levels after kisspeptin treatment[40]. 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[43] (or longer after
441 continuous kisspeptin administration), while it is known that LH pulses occur as frequently as
442 every < 10 min[44] in gonadectomized mice. This pulsatile inhibition upon GnRH neurons might be
443 mediated by dynorphin based on studies in sheep[45].

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/Tac2*KO 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 *Tac2*KO mice[40]. 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^{ARC} neurons (*Kiss1, Pdyn,*
460 *Tacr1 and Tacr3*). The expression of these genes is highly regulated (inhibited) by circulating sex
461 steroids[5,6]. 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^{ARC} neurons or, a more likely explanation, the pace-maker activity of Kiss1^{ARC}
472 neurons, which has been already demostrated[46]. 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

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

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

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Figure Legends:

Figure 1: Pubertal progression in male and female *Tac1/Tac2*KO mice. (**A,B**) Pubertal progression in males documented by preputial separation (PS) and body weight (BW) at the time of PS (**C**) (n ≥ 11/group). (**D,E**) Pubertal progression in females documented by vaginal opening (VO), BW at VO (**F**) and first estrus (**G**) (n ≥ 8/group). Estrous cyclicity during 30 days at age 3 months (**H**) and 8 months (**I**) (n ≥ 8/group). *p<0.05, **p<0.01. Student t test and One Way ANOVA followed by Newman Keuls *post hoc* test (H,I).

Figure 2: Fecundity test, gonadal histology and response to gonadectomy in male and female *Tac1/Tac2*KO mice. Litter size (A), parturition latency (B), testicular histology (C) and testicular weights (D) in WT and *Tac1/Tac2*KO males (n = 7/group)**.** Circulating LH levels before (basal) and 2 and 7 days after castration (E) (n = 10/group). In females, percentage of fertile females during 11 weeks of mating (F), litter size (G), parturition latency (H), ovarian histology (I), number of corpora lutea (CL) (J) and ovarian weights (K) in WT vs *Tac1/Tac2*KO females (n = 10/group). Circulating LH levels before (basal) and 2 and 7 days after ovariectomy (L). p<0.05, **p<0.01, *** p<0.001 Student t test. Different letters indicate significantly different values after a Two-Way ANOVA and repeated measures Fisher's *post hoc* test.

Figure 3: Pattern of LH pulsatility in gonadectomized WT and *Tac1/Tac2*KO mice. LH samples were collected every 10 min for 150 min in males and females 4 weeks after gonadectomy. Pattern of LH pulses, number of pulses/150 min and total secretory mass assessed by area under the curve (AUC) in males (n ≥ 3/group) (A-C) and females (n ≥ 6/group) (D-F). **p<0.01, Student t test. * denotes an LH pulses following the protocol described in the methods.

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

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

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

Statements

Statement of Ethics

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

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.

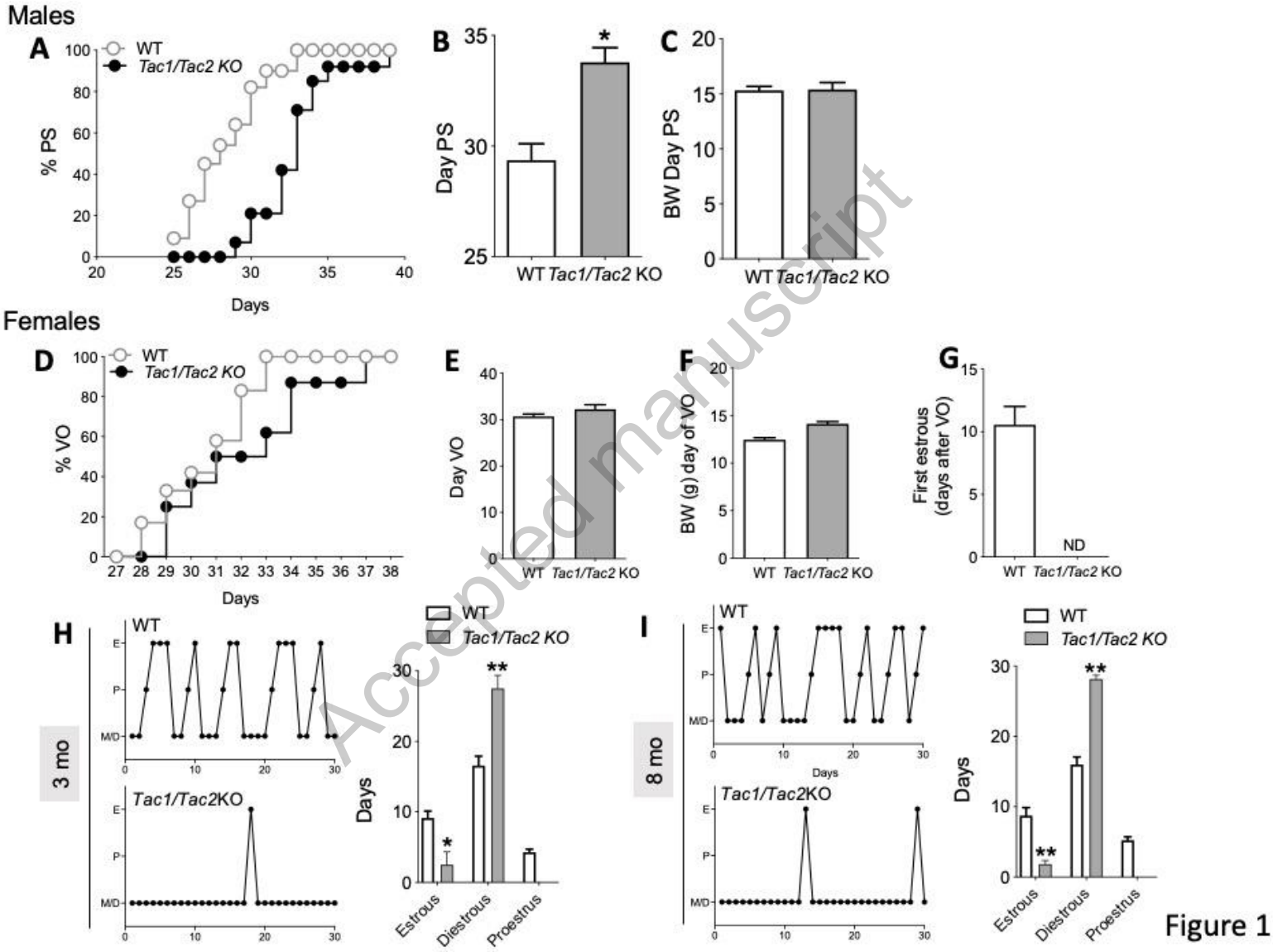
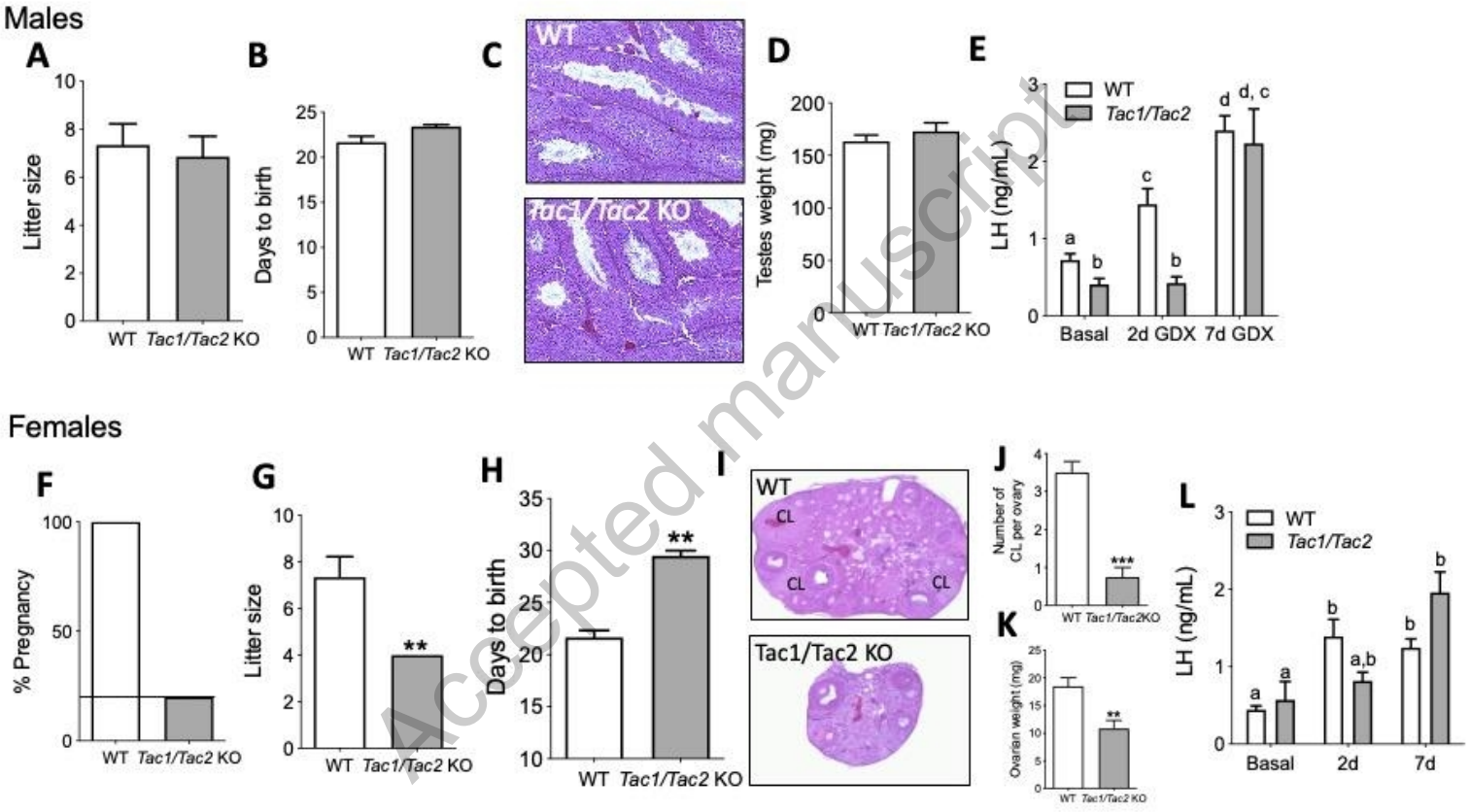


Figure 1



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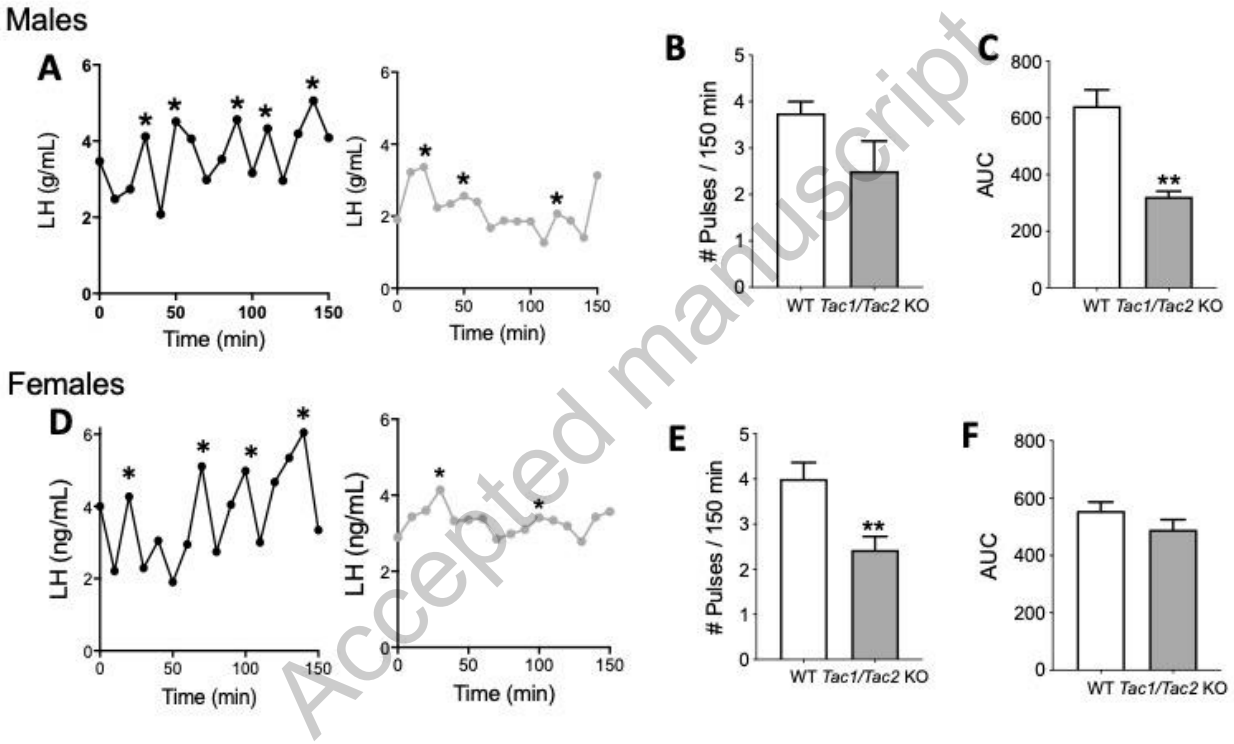
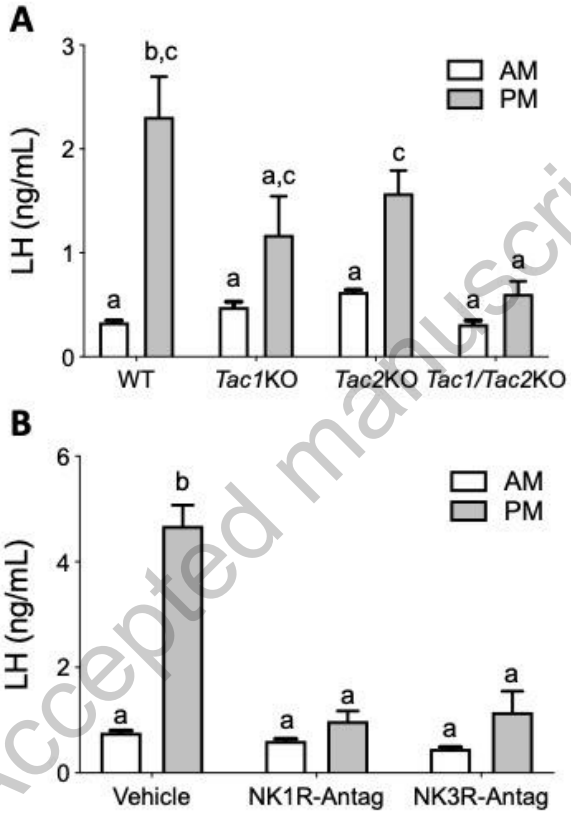


Figure 3



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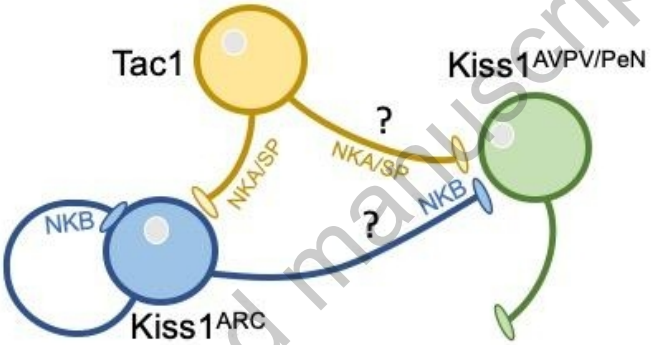
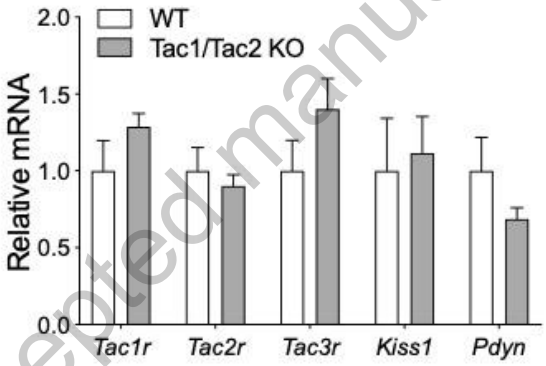


	Kiss1ARC		Kiss1AVPV/PeN	
	- E2/T	+ E2/T	- E2	+ E2/P
Endogenous Kiss1 activity				
+ Tachykinins				

Figure 5



Supplemental Figure 1