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.

Introduction

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

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

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

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

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

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

Materials and Methods

Mice. Wild-type (WT) female C57Bl/6 mice were purchased from Charles River Laboratories International, Inc. *Tac2* KO (knockout, KO) mice were obtained from Dr. Seminara (MGH, Boston, MA) (18). *Kiss1*KO were obtained from Dr. Richard Palmiter (University of Washington, Seattle, WA) (19). *Tac2*KO and *Kiss1*KO mice were compared to their WT littermates. All animal studies were approved by the Harvard Medical Area Standing Committee on the Use of Animals in Research and Teaching in the Harvard Medical School Center for Animal Resources and Comparative Medicine. 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 agonists for NK1R (GR73632), NK2R (GR64349) and NK3R (senktide), and the antagonists for NK3R (SB 222200) and NK1R (RP67580) were purchased from Tocris. Naloxone Hydrochloride (opioid receptor antagonist) and GnRH were purchased from Sigma Aldrich. Mouse kisspeptin-10 (Kp-10) was purchased from Phoenix pharmaceutical. All drugs were dissolved in saline (0.9% NaCl), except for SB 222200 and RP67580, which were dissolved in 5% DMSO. Doses and timing for hormonal analyses were selected on the basis of previous studies (9,20,21).

Experimental design

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

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

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

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

In this study, adult WT female mice were subjected to bilateral OVX under light isofluorane anesthesia, 1 week before pharmacological tests. Immediately after OVX, capsules filled with diluted crystalline of 17β-estradiol (E_2) or vehicle (sesame seed oil) were implanted subcutaneously (sc) via a small midscapular incision at the base of the neck (OVX+E_2). Silastic tubing (15 mm long, 0.078 in inner diameter, 0.125 in outer diameter; Dow Corning) was used for capsule preparation. A low dose of crystalline E_2 (50 µg/mL, in sesame oil) was used to fill the capsules which were sealed with silicone cement and allowed to cure overnight. The day before surgery, implants were washed twice for 10 min in changes of 100% ethanol and then placed in sterile physiological saline overnight.

First, we aimed to investigate the potential additive effect of the NK2R-Ag and senktide on LH secretion in the presence of sex steroids. To this end, LH levels were measured in WT OVX+E_2 females (n≥5 per group), 25 and 60 min after an icv injection of NK2R-Ag (600pmol), senktide (600pmol) or the co-administration of both drugs. Next, WT OVX+E_2 females (n≥5 per group) were pretreated with the NK3R antagonist SB222200 (7 nmol), 60 minutes prior to the icv injection of NK2R-Ag (600 pmol, senktinde or vehicle (0.9% NaCl). Blood samples were collected before SB222200 injection (basal) and at 25 and 60 minutes after injection of the agonists. Additionally, we further investigated the action of NK2R-Ag in the absence of NKB signaling using *Tac2*KO OVX+E_2 females and their corresponding WT littermate controls (WT OVX+E_2; n≥5 per group). Both groups of females were injected with NK2R-Ag (600 pmol) and blood samples were collected before and 25 and 60 min after injection. Finally, in order to evaluate whether the action of NKA requires kisspeptin to stimulate LH release, we used *Kiss1*KO OVX+E_2 females and their corresponding WT littermate controls (WT OVX+E_2; n≥5 per group) and LH levels were measured 25 min after icv injection of NK2R-Ag (600 pmol).

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

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

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

Study 4: *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 (n = 7) and *Tac2* KO (n = 9) ovary-intact females.

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 in **Table 1**.

In situ hybridization (ISH): To determine the presence of co-expression between *Tac2r* and *Tac1* mRNA in key areas (ventromedial nucleus, VMN; and ARC), dual fluorescence ISH was performed in tissue samples from OVX+sham and OVX+E_2 mice. We used probes for *Tac2r*-C1 and *Tac1*-C2 obtained from ACDBio and used the RNAscope method per their protocol (ACDBio). The brains were removed for ISH, fresh frozen on dry ice, and then stored at −80°C until sectioning. Five sets of 20-µm sections in the coronal plane were cut on a cryostat, from the

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

Hormone measurements: LH was measured by a sensitive sandwich ELISA for the assessment of whole blood LH concentrations (22). A 96-well high-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 [anhydrous], 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 (polyclonal goat anti-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 (22).

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 Tukey or Newman Kleus

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).

Author contributions

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

Results

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

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

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

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

Tacr2) is expressed in other neurons located in the ARC or ventromedial hypothalamus (VMH) and whether it colocalizes with Tac1 neurons in these areas as the source of SP and NKA. Our *in situ* hybridization (RNAscope) results showed that *Tacr2* is expressed in both ARC and VMH nuclei and colocalizes with virtually all Tac1 neurons in the VMH of adult WT mice regardless of the E_2 milieu (**Figure 2**).

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

We previously reported that the action of NKA and NKB on LH release is largely equivalent, i.e. both increase LH release in the presence of physiological circulating levels of E_2, but inhibit LH in the absence of sex steroids (9). It was, therefore, tentative to speculate that NKA could induce LH release through the stimulation of NKB given the absence of NK2R in Kiss1 and GnRH neurons. To test this hypothesis, first we co-administered NK2R and NK3R agonists inOVX + E_2 WT mice and observed that the increase in LH was similar in groups injected with an individual dose of each agonist or the combination of both (**Figure 3 A**), eliminating the possibility of an additive effect of NKA and NKB on LH release and suggesting a possible common pathway. Next, to evaluate if NKA requires NKB signaling to induce LH release, the LH response to NK2R-Ag was tested in the presence of an NK3R antagonist (**Figure 3 A**) or in NKB deficient (*Tac2*KO) mice (**Figure 3 B**). In both cases, NK2R-Ag was able to significantly stimulate LH release indicating that NK2R activation induces LH release independently of the presence of NKB or its receptor NK3R. However, NK2R signaling requires the presence of kisspeptin as *Kiss1*KO mice replaced with E_2 did not have any effect on LH release (WT Basal: 0.27 $\pm$ 0.06 ng/mL, WT NK2R-Ag: 0.57 $\pm$ 0.09 ng/mL; *p < 0.05; *Kiss1*KO Basal: 0.21 $\pm$ 0.02 ng/mL, *Kiss1*KO NK2R-Ag: 0.29 $\pm$ 0.04 ng/mL; not significant).

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

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

we showed that the inhibitory action of NK2R-Ag + senktide was similar to that of senktide alone, suggesting (as in the presence of E_2) that there is no additive effect of both tachykinins in the inhibition of LH (**Figure 4 A**). The use of the specific NK3R antagonist alone decreased LH in OVX animals, in line with recent literature showing that blockade of NK3R decreases LH pulsatility (23-26). However, co-administration of the NK3R antagonist and the NK2R-Ag failed to induce a further decrease in LH, suggesting that NK3R signaling is required for the inhibitory action of NKA in the absence of E_2 (**Figure 4 A**). Moreover, as previously described in rats, NK2R signaling requires dynorphin to inhibit LH (27), which is prevented after the blockade of the KOR and MOR using naloxone (**Figure 4 B**). Of note, naloxone alone also inhibited LH release in OVX mice, in line with our previous reports in OVX *Pdyn*KO and *Oprk1*KO mice (dynorphin KO and KOR KO mice, respectively) (28), suggesting that the absence of the inhibitory signal of dynorphin leads to a significant decrease in the ability of the mouse to secrete LH, probably due to disruption of the LH pulse generator mechanism (29). Next, we assessed the action of NK2R-Ag in the congenital absence of NKB (OVX *Tac2*KO mice) to further confirm the data obtained after NK3R blockade. Unexpectedly, we observed that the absence of NKB leads to a robust induction of LH release (**Figure 5 A**), revealing an action that is not present in WT OVX regardless of whether a functional NK3R is present or antagonized.

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

not significant). In order to assess whether this striking difference between the response to NK2R agonists after the pharmacological blockade of NK3R and the congenital absence of the NK3R ligand (NKB) is due to compensation in the expression of any of the ligand-receptor components of the tachykinin systems or in dynorphin, we evaluated the expression of these genes in the MBH of WT vs. *Tac2*KO female mice. We observed a significant increase in the expression of *Tacr3* (NK3R) in *Tac2*KO mice compared to controls (**Figure 5 C**).

Discussion

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

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

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

In this study we also addressed the question of whether the analogous action of NKA and NKB in the regulation of LH release (i.e. stimulation in the presence of E_2 and inhibition in its absence (9)) is due to the convergence of the NKA mechanism of action onto NKB signaling. Despite the aforementioned functional similarities, our data using an NK3R antagonist and *Tac2*KO mice after OVX or OVX and E_2 replacement, clearly demonstrate that the stimulatory action of NKA on LH is NKB *independent* but kisspeptin-dependent, suggesting the existence of a yet unknown population of NKA-responsive neurons that in turn activate Kiss1 neurons to induce kisspeptin/GnRH release (**Figure 6**). In contrast, NKA has been shown to inhibit LH via a mechanism which involves dynorphin in the rat (14,27) similar to what has been described for NKB in the absence of E_2 (39). Here, we show that the inhibitory action of NKA is weaker than the one exerted by NKB as observed 60 min after treatment, when NK2R agonist's action on the

inhibition of LH release is lost, while this inhibition reaches a maximal level for the NK3R agonist. Furthermore, we demonstrate that the inhibitory action of NKA is mediated by the activation of the NKB–dynorphin signaling pathway since blockade of both NK3R and KOR receptors prevented the inhibition of LH induced by the NK2R agonist. Importantly, the action of stimulating or inhibiting NK3R and KOR in both cases leads to the inhibition of LH release in the absence of sex steroids that is of equal magnitude 25 min after the treatment, likely as a consequence of the disruption of the GnRH pulse generator at the level of the ARC Kiss1 neuron. However, we unexpectedly observed that in the congenital absence of NKB, the NK2R agonist significantly stimulates LH release in the absence of E_2 (our present data in *Tac2*KO mice). These data suggest that the action of NKA is inherently stimulatory, NKB-independent and kisspeptin-dependent, as NK2R agonists did not induce any LH release in *Kiss1*KO mice regardless of the sex steroid milieu, unlike our recent findings on the kisspeptin-independent action of NKB (19). Thus, in *Tac2*KO mice, where NK3R and KOR are not blocked, the activity of the ARC Kiss1 neuron is significantly lower due to the absence of the stimulatory action of NKB, leading to lower basal LH levels after OVX (unpublished data and Figure 5). In this scenario, NKA is able to further stimulate Kiss1 neurons, leading to an increase in LH release. Whether this reflects heterodimerization of NK3R with NK1R, as we previously reported (9), heterodimerization with other receptors, or the convergence of intracellular pathways with NK3R's, remains unknown. Interestingly, the congenital absence of *Tac2* led to the compensatory rise of *Tacr3* and a noticeable trend to increase *Tacr1.* This is reminiscent of the increase in *Tacr2* observed in the absence of *Tac1* (*Tac1*KO mice (11)) and could also account, at least partially, for the increase in LH by NK2R agonists in *Tac2*KO mice regardless of the sex steroid milieu.

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

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

References

1. Lasaga M, Debeljuk L. Tachykinins and the hypothalamo-pituitary-gonadal axis: An update. *Peptides.* 2011;32(9):1972-1978.

2. Cao YQ, Mantyh PW, Carlson EJ, Gillespie AM, Epstein CJ, Basbaum AI. Primary afferent tachykinins are required to experience moderate to intense pain. *Nature.* 1998;392(6674):390-394.

3. Fergani C, Navarro VM. Expanding the Role of Tachykinins in the Neuroendocrine Control of Reproduction. *Reproduction.* 2016;153(1):R1-R14.

4. Arisawa M, De Palatis L, Ho R, Snyder GD, Yu WH, Pan G, McCann SM. Stimulatory role of substance P on gonadotropin release in ovariectomized rats. *Neuroendocrinology.* 1990;51(5):523-529.

5. Coiro V, Volpi R, Capretti L, Caiazza A, Marcato A, Bocchi R, Colla R, Rossi G, Chiodera P. Luteinizing hormone response to an intravenous infusion of substance P in normal men. *Metabolism.* 1992;41(7):689-691.

6. Traczyk WZ, Pau KY, Kaynard AH, Spies HG. Modulatory role of substance P on gonadotropin and prolactin secretion in the rabbit. *J Physiol Pharmacol.* 1992;43(3):279-297.

7. de Croft S, Boehm U, Herbison AE. Neurokinin B activates arcuate kisspeptin neurons through multiple tachykinin receptors in the male mouse. *Endocrinology.* 2013;154(8):2750-2760.

8. Rance NE, Bruce TR. Neurokinin B gene expression is increased in the arcuate nucleus of ovariectomized rats. *Neuroendocrinology.* 1994;60(4):337-345.

9. Navarro VM, Bosch MA, Leon S, Simavli S, True C, Pinilla L, Carroll RS, Seminara SB, Tena-Sempere M, Ronnekleiv OK, Kaiser UB. The integrated hypothalamic tachykinin-kisspeptin system as a central coordinator for reproduction. *Endocrinology.* 2015;156(2):627-637.

10. Hrabovszky E, Borsay BA, Racz K, Herczeg L, Ciofi P, Bloom SR, Ghatei MA, Dhillo WS, Liposits Z. Substance p immunoreactivity exhibits frequent colocalization with kisspeptin and neurokinin B in the human infundibular region. *PLoS One.* 2013;8(8):e72369.

11. Simavli S, Thompson IR, Maguire CA, Gill JC, Carroll RS, Wolfe A, Kaiser UB, Navarro VM. Substance p regulates puberty onset and fertility in the female mouse. *Endocrinology.* 2015;156(6):2313-2322.

12. Maguire CA, Song YB, Wu M, Leon S, Carroll RS, Alreja M, Kaiser UB, Navarro VM. Tac1 Signaling is Required for Sexual Maturation and Responsiveness of GnRH Neurons to Kisspeptin in the Male Mouse. *Endocrinology.* 2017.

13. Ruiz-Pino F, Garcia-Galiano D, Manfredi-Lozano M, Leon S, Sanchez-Garrido MA, Roa J, Pinilla L, Navarro VM, Tena-Sempere M. Effects and interactions of tachykinins and dynorphin on FSH and LH secretion in developing and adult rats. *Endocrinology.* 2015;156(2):576-588.

14. Sahu A, Kalra SP. Effects of tachykinins on luteinizing hormone release in female rats: potent inhibitory action of neuropeptide K. *Endocrinology.* 1992;130(3):1571-1577.

15. Steinhoff MS, von Mentzer B, Geppetti P, Pothoulakis C, Bunnett NW. Tachykinins and their receptors: contributions to physiological control and the mechanisms of disease. *Physiol Rev.* 2014;94(1):265-301.

16. Gianetti E, Tusset C, Noel SD, Au MG, Dwyer AA, Hughes VA, Abreu AP, Carroll J, Trarbach E, Silveira LF, Costa EM, de Mendonca BB, de Castro M, Lofrano A, Hall JE, Bolu E, Ozata M, Quinton R, Amory JK, Stewart SE, Arlt W, Cole TR, Crowley WF, Kaiser UB, Latronico AC, Seminara SB. TAC3/TACR3 mutations reveal preferential activation of gonadotropin-releasing hormone release by neurokinin B in neonatal life followed by reversal in adulthood. *J Clin Endocrinol Metab.* 2010;95(6):2857-2867.

17. Yang JJ, Caligioni CS, Chan YM, Seminara SB. Uncovering novel reproductive defects in neurokinin B receptor null mice: closing the gap between mice and men. *Endocrinology.* 2012;153(3):1498-1508.

18. True C, Nasrin Alam S, Cox K, Chan YM, Seminara S. Neurokinin B is critical for normal timing of sexual maturation but dispensable for adult reproductive function in female mice. *Endocrinology.* 2015:en20141862.

19. Fergani C, Leon S, Padilla SL, Verstegen AM, Palmiter RD, Navarro VM. NKB signaling in the posterodorsal medial amygdala stimulates gonadotropin release in a kisspeptin-independent manner in female mice. *Elife.* 2018;7:pii: e40476.

20. Navarro VM, Ruiz-Pino F, Sanchez-Garrido MA, Garcia-Galiano D, Hobbs SJ, Manfredi-Lozano M, Leon S, Sangiao-Alvarellos S, Castellano JM, Clifton DK, Pinilla L, Steiner RA, Tena-Sempere M. Role of neurokinin B in the control of female puberty and its modulation by metabolic status. *J Neurosci.* 2012;32(7):2388-2397.

21. Leon S, Barroso A, Vazquez MJ, Garcia-Galiano D, Manfredi-Lozano M, Ruiz-Pino F, Heras V, Romero-Ruiz A, Roa J, Schutz G, Kirilov M, Gaytan F, Pinilla L, Tena-Sempere M. Direct Actions of Kisspeptins on GnRH Neurons Permit Attainment of Fertility but are Insufficient to Fully Preserve Gonadotropic Axis Activity. *Sci Rep.* 2016;6:19206.

22. Steyn FJ, Wan Y, Clarkson J, Veldhuis JD, Herbison AE, Chen C. Development of a methodology for and assessment of pulsatile luteinizing hormone secretion in juvenile and adult male mice. *Endocrinology.* 2013;154(12):4939-4945.

23. George JT, Kakkar R, Marshall J, Scott ML, Finkelman RD, Ho TW, Veldhuis J, Skorupskaite K, Anderson RA, McIntosh S, Webber L. Neurokinin B Receptor Antagonism in Women With Polycystic Ovary Syndrome: A Randomized, Placebo-Controlled Trial. *J Clin Endocrinol Metab.* 2016;101(11):4313-4321.

24. Li SY, Li XF, Hu MH, Shao B, Poston L, Lightman SL, O'Byrne KT. Neurokinin B receptor antagonism decreases luteinising hormone pulse frequency and amplitude and delays puberty onset in the female rat. *J Neuroendocrinol.* 2014;26(8):521-527.

25. Nakamura S, Wakabayashi Y, Yamamura T, Ohkura S, Matsuyama S. A neurokinin 3 receptor-selective agonist accelerates pulsatile luteinizing hormone secretion in lactating cattle. *Biol Reprod.* 2017;97(1):81-90.

26. Noritake K, Matsuoka T, Ohsawa T, Shimomura K, Sanbuissho A, Uenoyama Y, Maeda K, Tsukamura H. Involvement of neurokinin receptors in the control of pulsatile luteinizing hormone secretion in rats. *J Reprod Dev.* 2011;57(3):409-415.

27. Kalra PS, Sahu A, Bonavera JJ, Kalra SP. Diverse effects of tachykinins on luteinizing hormone release in male rats: mechanism of action. *Endocrinology.* 1992;131(3):1195-1201.

28. Navarro VM, Gottsch ML, Chavkin C, Okamura H, Clifton DK, Steiner RA. Regulation of gonadotropin-releasing hormone secretion by kisspeptin/dynorphin/neurokinin B neurons in the arcuate nucleus of the mouse. *J Neurosci.* 2009;29(38):11859-11866.

29. Navarro VM. New insights into the control of pulsatile GnRH release: the role of Kiss1/neurokinin B neurons. *Front Endocrinol (Lausanne).* 2012;3:48.

30. Pinilla L, Aguilar E, Dieguez C, Millar RP, Tena-Sempere M. Kisspeptins and reproduction: physiological roles and regulatory mechanisms. *Physiol Rev.* 2012;92(3):1235-1316.

31. de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc Natl Acad Sci U S A.* 2003;100(19):10972-10976.

32. Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JS, Jr., Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG, Zahn D, Dixon J, Kaiser UB, Slaugenhaupt SA, Gusella JF, O'Rahilly S, Carlton MB, Crowley WF, Jr., Aparicio SA, Colledge WH. The GPR54 gene as a regulator of puberty. *N Engl J Med.* 2003;349(17):1614-1627.

33. Teles MG, Bianco SD, Brito VN, Trarbach EB, Kuohung W, Xu S, Seminara SB, Mendonca BB, Kaiser UB, Latronico AC. A GPR54-activating mutation in a patient with central precocious puberty. *N Engl J Med.* 2008;358(7):709-715.

34. Navarro VM, Fernandez-Fernandez R, Castellano JM, Roa J, Mayen A, Barreiro ML, Gaytan F, Aguilar E, Pinilla L, Dieguez C, Tena-Sempere M. Advanced vaginal opening and precocious activation of the reproductive axis by KiSS-1 peptide, the endogenous ligand of GPR54. *J Physiol.* 2004;561(Pt 2):379-386.

35. Navarro VM, Castellano JM, McConkey SM, Pineda R, Ruiz-Pino F, Pinilla L, Clifton DK, Tena-Sempere M, Steiner RA. Interactions between kisspeptin and neurokinin B in the control of GnRH secretion in the female rat. *Am J Physiol Endocrinol Metab.* 2011;300(1):E202-210.

36. Navarro VM, Ruiz-Pino F, Sanchez-Garrido MA, Garcia-Galiano D, Hobbs SJ, Manfredi-Lozano M, León S, Sangiao-Alvarellos S, Castellano JM, Clifton DK, Pinilla L, Steiner RA, Tena-Sempere M. Role of Neurokinin B in the Control of Female Puberty and Its Modulation by Metabolic Status. *J Neurosci.* 2012;In press.

37. Topaloglu AK, Reimann F, Guclu M, Yalin AS, Kotan LD, Porter KM, Serin A, Mungan NO, Cook JR, Ozbek MN, Imamoglu S, Akalin NS, Yuksel B, O'Rahilly S, Semple RK. TAC3 and TACR3 mutations in familial hypogonadotropic hypogonadism reveal a key role for Neurokinin B in the central control of reproduction. *Nat Genet.* 2009;41(3):354-358.

38. Young J, Bouligand J, Francou B, Raffin-Sanson ML, Gaillez S, Jeanpierre M, Grynberg M, Kamenicky P, Chanson P, Brailly-Tabard S, Guiochon-Mantel A. TAC3 and TACR3 defects cause hypothalamic congenital hypogonadotropic hypogonadism in humans. *J Clin Endocrinol Metab.* 2010;95(5):2287-2295.

39. Kinsey-Jones JS, Grachev P, Li XF, Lin YS, Milligan SR, Lightman SL, O'Byrne KT. The inhibitory effects of neurokinin B on GnRH pulse generator frequency in the female rat. *Endocrinology.* 2012;153(1):307-315.

Figure legends

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

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

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

Representative double label ISH depicting co-localization of *Tac1* (red) and *Tacr2* (green) mRNA. **(A)** VMH and **(C)** ARC of female WT C57Bl/6 mice after 1 week of OVX. **(B)** VMH and **(D)** ARC of female WT C57Bl/6 OVX mice after 1 week of E_2 replacement.

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

(A) LH release before (basal), 25 and 60 min after the icv injection of NK2R-Ag, senktide or the co-administration of both (600 pmol/5ul/icv) in WT OVX+E_2 females (n ≥ 5 per group). *p<0.05 vs. corresponding basal levels. (2 Way ANOVA followed by Tukey *post hoc* test). For SB222200 treated mice, LH levels before (basal) SB222200 (7 nmol5ul/icv) administration and at 25 and 60 minutes after injection of NK2R-Ag (600 pmol/5ul/icv), senktide (600 pmol/5ul/icv) or vehicle (0.9% NaCl/5ul/icv) in WT OVX+E_2 females (n ≥ 4 per group). *p<0.05 vs. corresponding basal

levels. # p<0.05 vs. corresponding control mice at the same time point (2 Way ANOVA followed by Tukey *post hoc* test). **(B)** LH before (Basal) and at 25 and 60 minutes after injection of NK2R-Ag (600 pmol/5ul/icv) in OVX+E_2 WT and *Tac2*KO females (n≥5 per group). *p<0.05 vs. corresponding basal levels (2 Way ANOVA followed by Tukey *post hoc* test).

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

(A) LH release before (basal), 25 and 60 min after icv injection of NK2R-Ag, senktide or the co-administration of both (600 pmol/5ul/icv) in WT OVX females (n≥5 per group). *p<0.05 vs. corresponding basal levels; # p<0.05 vs. NK2R-Ag at the same time point (2 Way ANOVA followed by Tukey post hoc test). For SB222200 treated mice, LH levels before (basal) SB222200 (7 nmol5ul/icv) injection and at 25 and 60 minutes after injection of NK2R-Ag (600 pmol/5ul/icv), senktide (600 pmol/5ul/icv) or vehicle (0.9% NaCl/5ul/icv) in WT OVX females (n≥5 per group). *p<0.05 vs. corresponding basal levels (2 Way ANOVA followed by Tukey *post hoc* test). **(B)** LH levels before (basal), 25 and 60 minutes after injection of naloxone (5mg/kg/100ul/ip) or vehicle (0.9% NaCl/100ul/ip) in WT OVX females (n≥5 per group). *p<0.05 vs. corresponding basal levels (2 Way ANOVA followed by Tukey *post hoc* test).

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

(A) LH levels before (basal) (vehicle or NK1R-Antg (2 nmol/5ul/icv)) and at 25 and 60 minutes after injection of NK2R-Ag (600 pmol/5ul/icv) or vehicle (0.9% NaCl/5ul/icv) in OVX *Tac2*KO females (n≥5 per group). *p<0.05 vs. corresponding basal levels (2 Way ANOVA followed by Tukey *post hoc* test). **(B)** LH levels before (basal) (vehicle or NK1R-Antg (2 nmol/5ul/icv)) and at 25 and 60 minutes after injection of NK1R-Ag (600 pmol/5ul/icv), or vehicle (0.9% NaCl/5ul/icv) in WT OVX females (n≥5 per group). *p<0.05 vs. corresponding basal levels (2 Way ANOVA followed by Tukey post hoc test); # p<0.05 vs. NK1R-Antg + vehicle injected mice levels. **(C)** Expression of *Tac1r, Tacr2, Tacr3, Kiss1* and *Pdyn* in the mediobasal hypothalamus of *Tac2*KO females (n=9) and their WT controls (n=7). *p<0.05 Student t-test.

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

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