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Hypothalamic Reproductive Endocrine Pulse Generator Activity Independent of Neurokinin B and Dynorphin Signaling

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Context: Kisspeptin-Neurokinin B-Dynorphin neurons are critical regulators of the hypothalamic-pituitary-gonadal axis. Neurokinin B (NKB) and dynorphin are hypothesized to influence the frequency of gonadotropin-releasing hormone (GnRH) pulses; whereas kisspeptin is hypothesized to be a generator of the GnRH pulse. How these neuropeptides interact remains unclear.
Objective: To probe the role of NKB in GnRH pulse generation and to dissect the interactions between NKB, kisspeptin, and dynorphin in humans and mice with a complete absence of NKB.
Design: Case/Control
Setting: Academic medical centers

Patients or Participants: Members of a consanguineous family bearing biallelic loss-of-function mutations in the gene encoding NKB and NKB deficient mice
Interventions: Frequent blood sampling to characterize neuroendocrine profile and administration of kisspeptin, GnRH, and naloxone, a non-specific opioid receptor antagonist used to block dynorphin.
Main Outcome Measure(s): Luteinizing hormone (LH) pulse characteristics
Results: Humans lacking NKB demonstrate slow LH pulse frequency which can be increased by opioid antagonism. Mice lacking NKB also demonstrate impaired LH secretion which can be augmented with an identical pharmacologic manipulation. Both mice and humans with NKB deficiency respond to exogenous kisspeptin.
Conclusion: The preservation of LH pulses in the absence of NKB and dynorphin signaling suggest that both peptides are dispensable for GnRH pulse generation and kisspeptin responsiveness. However, NKB and dynorphin appear to have opposing roles in the modulation of GnRH pulse frequency.

This study uses pharmacologic probes to demonstrate that endogenous GnRH-induced LH pulses can be generated in the absence of neurokinin B and dynorphin activity in humans and mice. .

Introduction

Despite nearly 50 years since the discovery of GnRH (1), understanding the factors that trigger GnRH neurons to drive the onset of sexual maturation and subsequently maintain reproductive function remains a challenge. Patients with idiopathic hypogonadotropic hypogonadism (IHH) are a key population to uncover these signals, as they have abnormal GnRH secretion/action (2, 3). Most IHH patients present as teens with delayed pubertal development and suffer life-long sexual infantilism and infertility if left untreated (2, 3).

Identification of the afferent pathways through which endogenous factors (e.g. gonadal steroids, stress hormones, and nutrient signals) and external cues (e.g. social cues and day length) regulate GnRH release have recently focused on the kisspeptin/neurokinin B/dynorphin system (4). Inactivating mutations in kisspeptin, neurokinin B (NKB), and their respective receptors cause IHH in humans and mice, implicating these neuropeptides in the generation of GnRH pulses (5-12). Dynorphin is thought to oppose this stimulatory activity by providing critical slowing of GnRH pulse generator activity in response to progesterone during the luteal phase of the menstrual cycle (13-15). These three neuropeptides coalesce in a population of neurons in the arcuate nucleus, KNDy (**K**isspeptin-**N**eurokinin B-**Dy**norphin) neurons, and are postulated to work in a coordinated fashion to synchronize the secretory activity of GnRH neurons to generate the pulses of GnRH secretion that are necessary to drive reproductive endocrine function (16-18).

Because biallelic loss-of-function mutations disrupt both copies of a gene, patients carrying such mutations (i.e. “human knockouts”) provide novel insights into the phenotypic consequences of gene disruption or loss. In this study, four sisters carrying biallelic, complete loss-of-function mutations in the gene encoding NKB (one of the key neuropeptides in KNDy neurons) underwent genotype-driven phenotyping. Despite an initial diagnosis of IHH, several sisters spontaneously recovered reproductive endocrine function in adult life. Studies were performed in both normal and neurokinin B-deficient family members as well as normal and neurokinin B-deficient mice to investigate the role of NKB in GnRH pulse generation and to dissect the interactions between NKB, kisspeptin, and dynorphin. Use of a combination of specific neuroendocrine probes revealed that the hypothalamus is capable of generating GnRH-

induced LH pulses despite genetic and pharmacologic antagonism of two of the three KNDy constituents, NKB and dynorphin.

Methods

Subjects and Eligibility Criteria

Five women from a single consanguineous family were recruited on the basis of their genotype (Table 1). Subjects were either reproductively normal (Subject 1; genotype *TAC3* c.61_61delG p.A21LfsX44 heterozygote) or carried a diagnosis of hypogonadotropic hypogonadism (Subjects 2-5; genotype *TAC3* c.61_61delG p.A21LfsX44 homozygote). The brothers and parents were not available for study participation. IHH was defined as hypogonadal sex steroid levels (estradiol <20 pg/mL in women) in the setting of low or normal gonadotropin levels at age ≥18 years and the absence of any identifiable medical condition that could cause hypogonadotropic hypogonadism. As in our previous report (19), reversal of IHH in women was defined as: 1) fertility without use of exogenous GnRH or gonadotropin therapy; 2) spontaneous menstrual cycling for at least 3 months in the absence of treatment; and/or 3) LH pulse frequency and amplitude within the normal range for women. Relapse after reversal was defined as again having hypogonadal sex-steroid levels (serum estradiol <20 pg/mL in women) and/or amenorrhea.

Subjects also participated in a genetics study. Patient DNA was screened for rare sequence variants (RSVs), defined as having a minor allele frequency of less than 1% in The Genome Aggregation Database (gnomAD), in genes known to cause IHH, as described previously (20, 21). Genes screened were *CHD7* (MIM 608892), *FGF8* (MIM 600483), *FGFR1* (MIM 136350), *GNRH1* (MIM 152760), *GNRHR* (MIM 138850), *HS6ST1* (MIM 604846), *ANOS1* (previously called *KAL1*, MIM 300836), *KISS1* (MIM 603286), *KISS1R* (MIM 604161), *NSMF* (previously called *NELF*, MIM 60813), *PROK2* (MIM 607002), *PROKR2* (MIM 607123), *TAC3* (MIM 162330), and *TACR3* (MIM 162332) by PCR amplification of exons followed by Sanger sequencing. RSVs were reported if they were predicted to be damaging by at least 2 out of 4 in silico prediction programs: PolyPhen-2 (22), SIFT (23), Mutation Taster (24), or Panther (25). The University of Pennsylvania Smell Identification Test (UPSIT) scores, from a 12-item smell test, were used to classify olfactory capabilities (26, 27).

Study Design

In 2010, the subjects with hypogonadotropic hypogonadism (Subjects 2, 3, 4, 5) underwent detailed neuroendocrine phenotyping in which blood sampling was performed every 10 minutes (q10 min) for 6-8 hours to map endogenous LH pulsations at the Wellcome Trust Clinical Research Facility, Cambridge, UK under the direction of Professor I. Sadaf Farooqi (Figure 1A).

In 2016, Subjects 1, 3, 4, and 5 were invited to participate in a second series of daytime studies at Massachusetts General Hospital (MGH) Clinical Research Center (CRC) to determine whether their endogenous LH pulse patterns could be modified by administration of GnRH, kisspeptin 112-121 (kp-10) and the non-specific opioid antagonist which blocks dynorphin, naloxone (NLX) (Figure 2A, Figure 3A, Figure 4A). To ensure that the pituitary gonadotropes would be in a state of readiness, Subjects 3 and 4 received exogenous pulsatile GnRH 25 ng/kg every 2 hours (q2h) by a Crono F portable infusion pump (Canè S.p.A, Turin, Italy) for 3 days prior to admission to the MGH CRC (28). Subject 5 had recent evidence of some neuroendocrine activity (yearly spontaneous bleeding) so she was not primed with pulsatile GnRH (Table 1).

Baseline studies: All subjects underwent q10 min blood sampling for at least 6 hours to evaluate endogenous GnRH-induced LH secretion during one of their visit days to the MGH CRC (Figure 1A, Figure 2A).

Kisspeptin boluses: After assessment of endogenous GnRH-induced LH secretion, subjects 3,4, and 5, received the administration of kp-10 0.24 nmol/kg intravenous bolus (IVB) as prior work by our group demonstrated that this dose consistently elicits GnRH-induced LH pulses of physiologic amplitude in healthy men and healthy luteal-phase women (29, 30) (Figure 2A). Subjects 4, 5 received subsequent kp-10 IVBs of 0.72 and 2.4 nmol/kg. Subjects 3, 4, and 5 then received 75 ng/kg IVB of GnRH at the conclusion of these studies, as our group has previously shown that this dose results in robust GnRH-induced LH responses in individuals with intact gonadotrope function (31).

Kisspeptin Infusion: In contrast to the IVB studies, Subject 3 returned to the CRC to participate in a second admission in which kp-10 was administered as a continuous infusion (9.5 nmol/kg/hr) for 12 hours to determine its effect on endogenous GnRH-induced LH pulsations. Similar to the IVB studies, blood samples were drawn q10 min and GnRH 75 ng/kg IVB was administered at study conclusion (Figure 3A).

Naloxone Infusion, Blocking Dynorphin: Subjects 4 and 5 returned to the CRC and received an NLX infusion (NLX 10 mg IVB, followed by infusion at 0.8 mg/hr) for 13 hours to determine the effect of blocking dynorphin signaling with opioid antagonism on endogenous LH pulses in the absence of NKB signaling. Midway through the infusion, kp-10 and GnRH boluses (kp-10 dose range: 0.24 to 2.4 nmol/kg, GnRH: 75 ng/kg) were administered to determine whether NLX administration might enhance the response to these peptides (Figure 4A). Again, blood samples were drawn q10 min for hormone measurements. Due to nursing error, subject 5 had the NLX infusion terminated early at hour 9.

Source of Peptides

Kisspeptin 112–121, the 10-amino-acid isoform of kisspeptin (corresponding to amino acids 112-121 of the pre-prohormone), and GnRH were synthesized using good manufacturing practices by NeoMPS (PolyPeptide Laboratories, San Diego, CA). NeoMPS provided kisspeptin 112-121 under contract to the Eunice Kennedy Shriver National Institute of Child Health and Human Development. Naloxone was ordered from Hospira (Lake Forest, IL).

Human Laboratory Assays

LH for each sample and estradiol on 2-hour pools were measured by direct immunoassay using the automated Abbott ARCHITECT system (Abbott Laboratories, Inc., Abbott Park, IL) as previously described (28). Estradiol was measured by a 2nd generation immunoassay traceable to mass spectrometry-based assays for the 2010-2011 studies and by Elecsys (Roche Diagnostics, Indianapolis, IN) for 2016 studies (32, 33).

Assessment of Pulsatile LH Release in Peripubertal and Adult Tac2 Knockout Mice

Tac2$^{+/-}$ breeding pairs were generated by the Texas A&M Institute for Genomic Medicine (College Station, TX) and genotyped (34). All mice were generated and maintained on a Sv129/C57BL/6 hybrid background and group housed (three to five per cage) at the Brigham and Women's Hospital in a temperature- and light-controlled environment with lights on from 0600–1800 h and food and water provided ad libitum. Mice were handled daily for two to six weeks prior to the experiment to allow acclimation to sampling conditions.

Changes in LH secretion was assessed in sexually maturing (6-week-old) and adult (16-week-old) intact and ovariectomized (OVX) *Tac2* knockout (KO) female mice and control (wild-

type; WT) littermates (n=4-5 per group). Since *Tac2* in mice encodes for NKB in humans, these mice are lacking NKB. Pulsatile measurements of LH secretion were assessed by repeated blood collection through a single incision at the tip of the tail. The tail was cleaned with saline then four ul blood was taken at each time point from the cut tail 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. For kp-10 administration studies, thirty-six sequential blood samples were collected over a 6-hour sampling period. At 170 min of sampling (or 180 min of sampling for peripubertal *Tac2* knockout mice), mice were injected with mouse kp-10 intraperitoneally (7.5 nmol/100 ul saline; Phoenix Pharmaceuticals). For NLX administration studies, thirty sequential blood samples were collected over a 5-hour sampling period from WT and *Tac2* KO mice. WT and *Tac2* KO mice were OVX'd to increase the frequency and amplitude of LH pulses to better determine the action of dynorphin removal in the generation of LH pulses. At 120 min of sampling, mice were injected with NLX intraperitoneally (5 mg/kg/100 ul saline; Sigma Aldrich).

Data Analysis

Human Pulse Analysis: LH pulses were identified using a validated modification of the Santen and Bardin method (35, 36) augmented by a deconvolution algorithm (29). Pulse amplitude of kp-10-induced or GnRH- induced LH pulses was calculated as the difference between time 0 of kp-10 or GnRH administration and the peak of the pulse.

Mouse Pulse Analysis: LH pulses were identified using a custom-made MATLAB code that reads the LH pulse data gathered by LH sandwich ELISA. The code includes a loop that determines a pulse based on if: a) the height of an LH value is 20% greater than the heights of either of the 2 previous values as well as 10% greater than the height of the following value; b) the peak at the second-time interval (i=2) is >20% greater than the single value that comes before it to be considered a pulse.

Statistics: Paired two-way t-tests were used to assess changes in mean LH, LH amplitude (nadir to peak of an LH pulse) and FSH at baseline, as defined in methods above, as compared to responses to neuropeptide interventions. All values are reports as mean ± standard deviation, unless otherwise noted.

Study Approval

All human studies were approved by the Institutional Review Board of MGH/Partners Healthcare, or by the Local Regional Ethics Committee of Cambridge, United Kingdom. All subjects gave written informed consent prior to inclusion in the studies. For the mouse studies, the Brigham and Women's Hospital Institutional Animal Care and Use Committee approved all procedures.

Results

Study Subjects Initial Clinical Presentation and Subsequent Course

Subject 1 had a normal timing of menarche, normal menstrual cycles, and spontaneous pregnancy (Table 1). Her sisters, Subjects 2, 3, 4, and 5, presented at 13-15 y with primary amenorrhea and received estrogen therapy to induce secondary sexual characteristics. Because of the lack of spontaneous sexual maturation by age 18**,** normal MRI, and low gonadotropins, Subjects 2, 3, 4, and 5 all received a diagnosis of IHH (Table 1). None of the sisters are anosmic. Three of the four IHH sisters demonstrated reversal of their hypogonadotropism between 22-28 y as evidenced by pregnancy without fertility medications (Subjects 3 and 4) and regular spontaneous menstrual cycles (Subject 5). However, reversal was not permanent and at the time

of the physiologic studies, subjects 3, 4, and 5 had reverted to a state of hypogonadotropic hypogonadism (Table 1).

Genetics

Sequencing of candidate genes revealed that Subject 1 (normal timing of puberty and normal menstrual cycles) is heterozygous for a deletion of a single nucleotide in the gene encoding NKB (*TAC3)* (c.61_61delG p.A21LfsX44). This base pair deletion leads to a frameshift mutation and a premature stop codon, in the pre-prohormone prior to the NKB sequence, that would be predicted to result in nonsense-mediated decay. Even if the transcript were to escape nonsense-mediated decay, the frameshift mutation would disrupt the portion of the pre-prohormone that is processed to produce the decapeptide known as NKB. Subjects 2, 3, 4, and 5, all with hypogonadotropic hypogonadism, are homozygous for this frameshift mutation. This mutation is novel and not found in gnomAD, a normative database containing 123,136 exomes and 15,496 genomes (21). Notably, there are no individuals homozygous for any protein-truncating mutations in *TAC3* in gnomAD. This family harbors no other mutations in genes known to cause IHH.

Baseline Studies: Slow LH Pulse Frequency Characterizes IHH Individuals Without Neurokinin B

At the time of these baseline studies, the IHH sisters (Subjects 2,3,4 and 5) were amenorrheic with low but detectable serum estradiol levels and low progesterone levels off hormonal medications (Table 1, Figure 1B). All subjects with IHH had evidence of an enfeebled but organized GnRH pulse generator, as evidenced by low-frequency LH secretory events (for comparison in the physiologic early follicular phase which is characterized by low estradiol, low progesterone: LH frequency, 7.0 ± 1.8 pulses/12 h; LH amplitude, 2.3 ± 1.0 IU/L [mean ± 2 SD]) (37, 38). In Subjects 2, 4, and 5, one pulse was observed in the sampling interval (7-8 hours; mean LH amplitude 1.5±0.8 mIU/mL) (Figure 1B). In Subject 3, no pulses were observed during the study. In addition, the LH levels of Subjects 3, 4, and 5 demonstrated slow decay at the beginning of the sampling interval, suggesting that an LH secretory event had occurred before the start of the study. Thus, all subjects demonstrated an abnormally low frequency of LH secretory events. Upon repeat testing in 2016, study subjects (Subjects 3, 4, 5) again were amenorrheic with low but detectable estradiol levels off hormonal medications. All studies recapitulated the same endogenous LH patterns observed in 2010, with low-frequency LH secretory events and a mean LH amplitude of 1.3 ± 1.1 mIU/mL (Figure 2B).

In contrast, Subject 1, the healthy sister with a heterozygous protein truncating variant in *TAC3*, underwent blood sampling on Day 4 of the menstrual cycle (early follicular phase; EFP). She exhibited 11 LH pulses in 12 hours with a mean LH pulse amplitude of 0.46 ± 0.25 mIU/mL (Figure 1C) (healthy early follicular phase women: frequency, 7.0 ± 1.8 pulses/12 h; amplitude, 2.3 ± 1.0 IU/L [mean ± 2 SD]) (19, 20).

Kisspeptin Boluses: IHH Individuals without NKB Respond to Kisspeptin

All subjects responded to kisspeptin with an LH pulse (Figure 2B). Two study subjects received three kisspeptin boluses and demonstrated an LH pulse following kisspeptin in 5 of the 6 boluses. The one exception occurred when kisspeptin was administered immediately following an endogenous LH peak resulting in a prolonged single peak (Figure 2B, Subject 5). Consistent with this responsiveness, all subjects demonstrated adequate pituitary priming, indicating no pituitary defect that could impair kisspeptin responsiveness (LH pulse amplitude following GnRH administration: Subject 3: 1.6 mIU/mL, Subject 4: 5.1 mIU/mL, Subject 5: 3.0 mIU/mL).

Kisspeptin Infusion: No Pulsatile LH Secretion

Subject 3 received a kp-10 infusion (9.5 nmol/kg/hr) for 12 hours and no LH pulses were detected. There was a modest increase in mean LH during the infusion (baseline: 0.46 ± 0.24 mIU/mL; kp-10 infusion: 0.63 ± 0.08 mIU/mL; $p<0.0001$) (Figure 2B & 3B). Mean FSH levels also increased as compared to baseline (baseline: 1.9 ± 0.2 mIU/mL; kp-10 infusion: 2.4± 0.1

mIU/mL; p<0.001). After the kp-10 infusion, Subject 3 received an IVB of GnRH resulting in an LH pulse of comparable amplitude to that observed in baseline study the prior day (baseline, 1.6 mIU/mL; after kp-10 infusion, 2.5 mIU/mL).

Naloxone Infusion: Blocking Dynorphin with Naloxone Increases LH & FSH Secretion and LH Pulse Frequency, but Does Not Amplify Kisspeptin-Induced LH Pulses

Subjects 4 and 5 received the non-selective opioid antagonist, NLX, as well as escalating boluses of kisspeptin (0.24, 0.72, 2.4 nmol/kg) to determine the effect of blocking dynorphin signaling on endogenous and kisspeptin-stimulated LH secretory patterns. Both studies demonstrated increased mean LH levels during NLX infusion as compared to baseline (Subject 4 – baseline: 1.44 ± 0.76 mIU/mL, NLX: 2.82 ± 0.54 mIU/mL, p<0.00001; Subject 5 – baseline: 0.6 ± 0.25 mIU/mL, NLX: 1.1 ± 0.37 mIU/mL, p<0.00001, across matched time points) (Figure 2B, 4B). For the study subject in which a complete LH sampling on and off NLX infusion allowed comparison, Subject 4, LH pulse frequency increased from one pulse in 6 hours (Figure 2B) to four pulses in 6 hours (Figure 4B). Mean FSH levels also increased as compared to baseline (Subject 4 – baseline: 3.7 ± 0.3 mIU/mL, NLX: 5.0± 0.9 mIU/mL; p<0.01; Subject 5 – baseline: 3.3 ± 0.3 mIU/mL, NLX: 5.1± 0.1 mIU/mL; p<0.0001). There was no consistent change in LH pulse amplitude (Subject 4 – baseline: 2.59 mIU/, NLX: 0.45 ± 0.29 mIU/mL; Subject 5 – baseline: 0.82 mIU/mL, NLX: 1.22 and 1.39 mIU/mL). NLX infusions, which block dynorphin by inhibiting opioid tone, increase gonadotropin secretion and improve LH pulse frequency in individuals with IHH due to loss of NKB signaling.

Subjects 4 and 5 also received escalating boluses of kp-10 (0.24, 0.72, 2.4 nmol/kg) which were followed by an LH pulse, recapitulating results seen off NLX (Figure 2B, 4B). There was no significant difference in the change in kisspeptin-induced LH response on or off NLX and there was no clear dose-response relationship; although the small number of boluses at each dose limited the ability to assess such a relationship.

Kisspeptin Boluses Stimulate LH Release in Peripubertal and Adult WT and NKB-deficient (Tac2 KO) Mice

To corroborate the findings in IHH patients, we conducted experiments in *Tac2* KO and WT control female mice. Peripheral administration of kp-10 elicited a robust increase in LH in all animal groups regardless of age and genotype. Interestingly, peripubertal *Tac2* KO female mice, lacking NKB, displayed a higher magnitude of LH release (5.29 ± 0.43 ng/ml, n=5) than control females (2.67 ± 0.48 ng/ml, n=5; p<0.01) (Figure 5). However, LH returned to baseline faster in *Tac2* KO mice (52 ± 3.72 min after injection, n=5) than in WT control (68 ± 3.72 min, n=5; p<0.01). Adult WT mice displayed the expected LH pulse in response to kp-10, while the *Tac2* KO mice that responded to kp-10 showed a bi-phasic response, displaying two overlapping peaks of LH (Figure 4). In both adult groups, the induction of LH release appeared more sustained than in peripubertal mice (peripubertal WT: 68 ± 3.742 min, n=5 vs adult WT142.5 ± 4.78 min after injection, n = 4, p < 0.0001; peripubertal *Tac2* KO: 52 ± 3.742, n=5 vs adult *Tac2* KO 156.7 ± 3.33 min, n = 3, p=0.07).

Naloxone Increases Pulsatile LH Release in Adult OVX WT and Tac2 KO Mice

To determine the role of the opiatergic (dynorphin) influence on kisspeptin signaling in the absence of NKB, we examined the effects of NLX, which blocks dynorphin, on LH secretion. Peripheral administration of NLX 5 mg/kg induced an increase in LH in both WT (Figure 6 A-C) and *Tac2* KO female mice (Figure 6 D-E) within 20 min of administration (WT: 20 min pre-NLX, 2.37 ± 0.59, n=4 vs 20 min post NLX, 4.31 ± 0.32, n=4; p<0.05. *Tac2* KO: 20 min pre-NLX, 0.31 ± 0.06, n=4 vs 20 min post NLX, 1.22 ± 0.29, n=4, p<0.05).

After NLX administration, WT mice responded with an increase in the duration of the following LH pulse post-NLX administration (pre-NLX: WT 25± 2.67 min, n = 3; *Tac2* KO

23.33 ± 2.10 min, n = 3, p= 0.13; post-NLX: WT: 83.33±12.02 min, n = 3; *Tac2* KO: 30±5.77 min, n = 3; p<0.01) (Figure 6A). In addition, the increase in duration in the post-NLX LH pulse was accompanied by a pronounced and longer inter-pulse interval in WT mice (WT inter-pulse interval pre-NLX 25.38±1.83 min; WT inter-pulse interval post-NLX 46.67±3.33 min, p<0.0002).

Tac2 KO animals displayed a markedly reduced LH baseline and number of pulses than in OVX controls (0-1 LH pulses in 120 min pre-NLX). The administration of NLX induced a robust LH pulse that occurred 20 min after treatment in all cases, with a peak that reached a two-fold increase compared to baseline (pre-NLX: 0.31 ± 0.06 mIU/mL; post-NLX: 1.2 ± 0.28 mIU/mL, p<0.02). While the limited number of LH pulses precluded an analysis of inter-pulse intervals; data suggest that NLX did not increase the duration of the LH pulse (pre-NLX *Tac2* KO 23.33 ± 2.10 min, n = 3, post-NLX: *Tac2* KO: 30±5.77 min, n = 3, p>0.05) (Figure 6 D-E).

Discussion

In this study, 1) naturally occurring loss-of-function mutations in the gene encoding NKB in a consanguineous family, 2) biochemical phenotyping, and 3) provocative challenge testing were all employed to explore the physiologic architecture underlying GnRH pulse generation in the hypothalamus of mice and humans. Although IHH patients carrying mutations in the gene encoding the NKB receptor (*TACR3*) are not uncommon, only one family with a genetic mutation leading to a complete loss of NKB (*TAC3*) has been reported in the literature to date (39). In this series of genotype-driven physiologic investigations, the genetic loss of NKB provided a key backdrop for baseline and provocative detailed neuroendocrine phenotyping.

Most patients with IHH have a lack of GnRH-induced LH pulsations (2). In this study, four sisters with IHH bearing homozygous loss of function mutations in *TAC3* demonstrated a unique neuroendocrine pattern of well-articulated, but infrequent, LH pulses; this pattern showed remarkable fidelity across all 4 sisters and is similar to another published report (40). In parallel, ovariectomized *Tac2* mutant mice demonstrated reduced LH pulse frequency compared to WT controls. On the one hand, the slow frequency of LH pulses speaks to the important role of NKB as a driver of normal GnRH-induced LH pulse frequency. NKB signaling has been specifically associated with GnRH pulse frequency (39) and NKB receptor antagonists have recently been shown to reduce LH pulses in post-menopausal women and patients with polycystic ovarian syndrome (41, 42). The endogenous opioid, dynorphin, potentially “unrestrained” by the pathophysiologic absence of NKB, may also have contributed to the lengthy LH inter-pulse interval (43). However, the observation of any LH pulses, even infrequent ones, clearly demonstrates that NKB is not essential for GnRH-induced LH pulse generation per se. The identity of the drivers of these low-frequency LH secretory events, (kisspeptin, GnRH, other tachykinins, or factors yet to be discovered) requires further study (44-46).

Although loss-of-function mutations in both kisspeptin and NKB signaling have been associated with hypogonadotropic hypogonadism, there appears to be greater complexity in the phenotype associated with deficiency of NKB signaling compared to that of kisspeptin (47). Subjects 3, 4, and 5 experienced reversal of their hypogonadotropic phenotype as evidenced by their ability to have spontaneous menstrual cycles and fertility in the absence of any medications. It is tempting to speculate that their low frequency LH pulses observed in both 2010 and 2016 are related to their phenotypic reversal, i.e. an intact GnRH pulse generator, even if slow, can be sped up leading to reversal under the right circumstances. Additional studies, perhaps using

opioid antagonists such as in this study, would be required to reach that conclusion with greater certitude.

The most remarkable finding of this study was the increase in LH levels during the NLX infusion in subjects with IHH. To date, the ability to stimulate *endogenous* GnRH-induced LH pulsations that mimic normal physiology in patients with IHH has been non-existent. The observation of a normal LH pulse frequency in the absence of both a key driver for kisspeptin-induced-GnRH-induced LH pulsations (NKB) and a key inhibitor (dynorphin) demonstrate that both NKB and dynorphin are dispensable for GnRH pulse generation and termination. We have previously postulated that the reproductive cascade has several potential pulse generators that are capable of "standing in" when upstream inputs are dysfunctional. Possibilities include, but are not limited to 1) pulsatile kisspeptin secretion from KNDy neurons in the absence of NKB/dynorphin autofeedback (48), 2) other tachykinins that substitute in for NKB (45), 3) pulsatile kisspeptin secretion from non-KNDy neurons (49), or 4) kisspeptin-independent pulsatile GnRH secretion (50).

Considerations regarding LH pulses include the observation that Subject 4 appeared to have a more pronounced response to NLX than Subject 5. Subject 4 underwent pituitary priming with exogenous GnRH and Subject 5 did not, which may have amplified any effect of NLX on the LH response in Subject 4. Subject 4 had also been receiving intermittent hormone replacement therapy which may have enhanced endogenous kisspeptin action on GnRH release. This speculation is based on observations showing that periodic exposure to estradiol appears to be essential for kisspeptin action in female non-human primates (51). The ability to generalize these findings beyond patients with NKB pathway mutations is unclear. Prior attempts to stimulate the reproductive axis in IHH patients (of unknown genotype) using NLX were not successful (52).

In synchrony with the human observations, LH levels increased during NLX injection in OVX WT and *Tac2* mutant mice. LH pulse amplitude was clearly increased; an increase in LH pulse frequency could not be assessed due to the limited duration of the NLX injection as well as limitations of blood sampling. These findings are consistent with previous observations that NLX increases LH levels and/or pulse frequency in healthy humans and humans with hypothalamic amenorrhea, an acquired form of hypogonadism (15, 53, 54). Furthermore, these findings extend the observations regarding the effects of dynorphin on GnRH pulse termination reported in sheep, demonstrating treatment with a kappa-opioid receptor specific antagonist can prolong NKB-stimulated LH pulses (55, 56). Taken together, these studies suggest the need for further dissection of cellular events that lead to NLX's impact on GnRH pulse generation in the presence and absence of NKB.

In prior studies, the inability of the same dose of kp-10, which effects a robust GnRH-induced LH response in healthy men and luteal-phase women, to bring about any effect in IHH patients across a range of genotypes suggested that the functional capacity of the GnRH neuronal network is fundamentally impaired in patients with IHH (28). In contrast to these previous observations in IHH patients with genotypes other than *TAC3* or *TACR3*, Subjects 3, 4, and 5 responded to kp-10 IVB (28). Here, the low frequency pulses and the ability to respond to exogenous kp-10 administration suggest that the GnRH neuronal circuitry necessary for pulse generation remains intact in patients lacking NKB. However, the ability to respond to kp-10 with LH pulses was observed only in the setting of IVB administration, and not a continuous infusion, as has been reported by others (40). Differing doses of kp-10, LH assays and LH pulse algorithms may account for this discordance.

Comparing NKB human "knock-outs" with the female non-human primate receiving pharmacologic blockade of NKB receptor signaling reveals parallels in the development of hypothalamic brain circuitry. In rhesus monkeys, reciprocal signaling mechanisms between kisspeptin and NKB neurons appear to be established over the course of sexual maturation. Thus, kisspeptin-induced GnRH secretion is possible in the presence of the NK3R antagonist, SB222200, in the prepubertal state, but is blocked in the presence of SB222200 in the pubertal state (57). Furthermore, female pubertal monkeys require the presence of circulating estradiol to respond to kp-10; whereas pre-pubertal monkeys do not (51). In the current study, the observation that hypogonadal female patients without endogenous NKB are capable of responding to kp-10 suggests that they too have intact hypothalamic circuitry akin to that of a prepubertal monkey.

As in the human model, the mice lacking NKB (encoded for in mice by *Tac2*), in both the peripubertal and adult period, responded to kp-10 with robust GnRH-induced LH pulses. Because *Tac2* KO mice have an impaired reproductive axis in early life which then normalizes in adulthood, both phases of reproductive life were examined (34). In the current studies, the kp-10 stimulated GnRH-induced LH pulse amplitude was higher in *Tac2* KO mice than WT mice and changed over time, appearing as a single pulse in sexually immature animals but biphasic in adulthood. Substance P is known to stimulate LH release and, in female animals in the setting of low sex steroids, does so during the upswing of an LH pulse which could give the appearance of a biphasic pulse (45, 58-60). It has been hypothesized that the *Tac2* KO mouse overcomes its delay in sexual maturation and establishment of normal estrus cycles due to other tachykinin inputs. As the substance P receptor is directly expressed on GnRH neurons, further research into its effect on the morphology of the LH pulse may reveal ways in which kisspeptin's action can be augmented in mice lacking NKB.

In this series of studies, the use of a human genetic "knock-out" for NKB reveals a robust GnRH pulse generator in the absence of NKB and dynorphin signaling. Furthermore, it demonstrates the antagonistic relationship between stimulatory NKB and inhibitory dynorphin in modulation of endogenous GnRH pulse frequency. Kisspeptin is capable of stimulating GnRH-induced LH release in humans and mice lacking NKB. Further studies will be required to explore the role of antagonism of endogenous opioids in hypogonadotropic states. Nevertheless, the finding in this study that endogenous kisspeptin signaling alone is sufficient for GnRH pulse generation in human patients, demonstrates the human relevance of findings from Herbison and his colleagues that optogenetic excitation of selective kisspeptin neurons induces GnRH pulses in mice (17, 18, 61). Collectively, this knowledge suggests that there may be a role for opioid antagonism in the treatment of patients with reproductive disorders due to NKB deficiency and that this may also extend to those reproductive disorders characterized by slow GnRH pulse frequency.

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Figure 1: Baseline Neuroendocrine Profiling. A. Study schema; B. Study subjects with IHH who underwent 8 hr sampling between 2010-2011; C. Healthy sister in early follicular phase (EFP). E2 = estradiol, P= progesterone, LH = luteinizing hormone.

Figure 2: Baseline Studies with Response to Kisspeptin and GnRH. A. Study schema; B. Study subjects. Arrows indicated luteinizing hormones pulses detected by the algorithm. K = kisspeptin-10 by intravenous boluses and subscript indicates the dose 1=0.24 nmol/kg, 2= 0.72 nmol/kg, 3 = 2.4 nmol/kg. G= GnRH IVB 75 ng/kg. E2 = estradiol, FSH = follicle stimulating hormone, LH = luteinizing hormone.

Figure 3: Response to Kisspeptin Infusion and GnRH. A. Study schema; B. Study subject. Arrows indicated luteinizing hormones pulses detected by the algorithm. G= GnRH IVB 75 ng/kg. E2 = estradiol, FSH = follicle stimulating hormone, LH = luteinizing hormone.

Figure 4: Neuropeptide Administration with Response to Kisspeptin and GnRH. A. Study schema; B. Study subjects. Arrows indicated luteinizing hormones pulses detected by the algorithm. K = kisspeptin-10 by intravenous boluses and subscript indicates the dose 1=0.24

nmol/kg, 2= 0.72 nmol/kg, 3 = 2.4 nmol/kg. G= GnRH IVB 75 ng/kg. E2 = estradiol, FSH = follicle stimulating hormone, LH = luteinizing hormone.

Figure 5: Kisspeptin administration to Tac2 Knock-out mice and littermate controls across sexual development. Dashed line = kisspeptin administration. Luteinizing hormone values are mean ± SEM for each timepoint.

Figure 6. LH pulse profile (A and D) and the effects of naloxone (NLX) (B, C, E, and F) in adult OVX WT and Tac2 Knock-out mice. A and D: LH pulses 120 min before NLX injection, and 180 min after NLX injection; NLX injection indicated by arrows. Arrowheads indicate the LH pulses. B and E: Changes in LH secretion (mean ± SEM) 60 min before and 120 min after NLX in WT and OVX Tac2KO mice, respectively. C and F: The effects of NLX treatment on LH release are also shown as mean ± SEM from 20 min before (Pre NLX) and 20 min after NLX injection (Post NLX). * $P < 0.05$, Student t test.

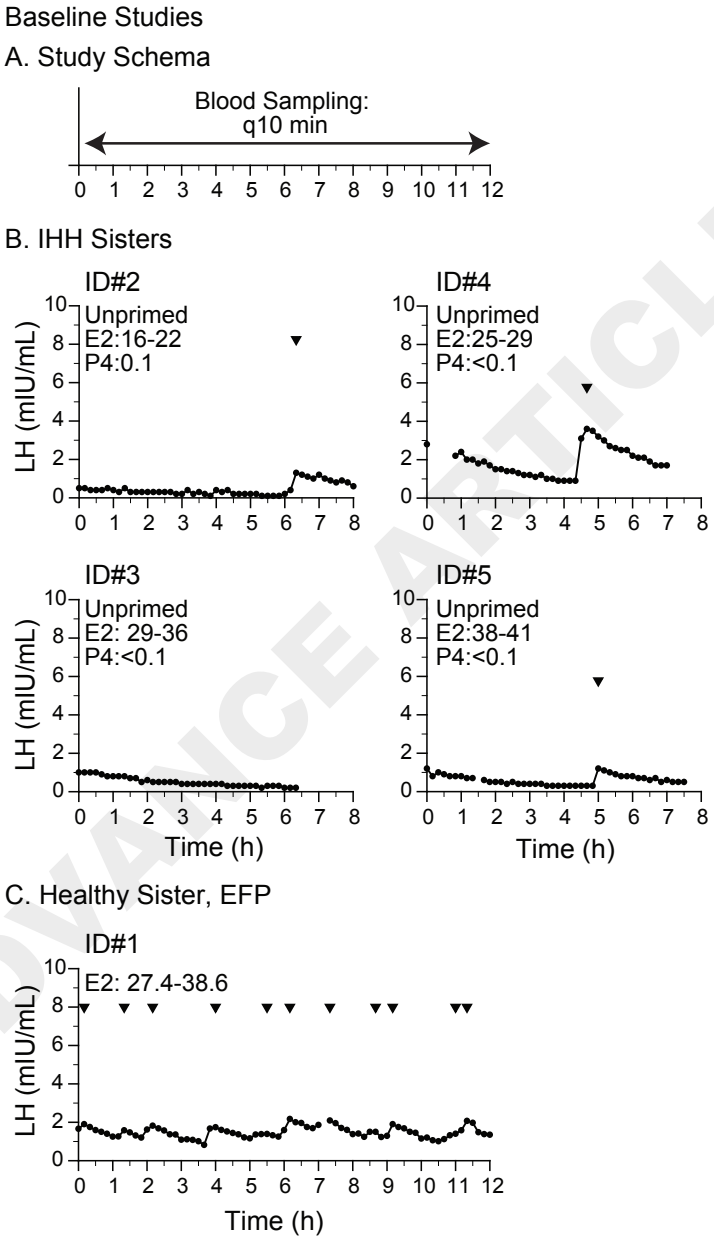
Table 1: Study Subject Characteristics. FSH = follicle stimulating hormone, LH = luteinizing hormone, E2 = estradiol, IVB = intravenous bolus, kiss = kisspeptin, GnRH = gonadotropin stimulating hormone, US = transvaginal ultrasound, HRT = hormone replacement therapy, MPA = medroxyprogesterone acetate, CC = clomiphene citrate, SAB = spontaneous abortion, OCPs = oral contraceptive pills

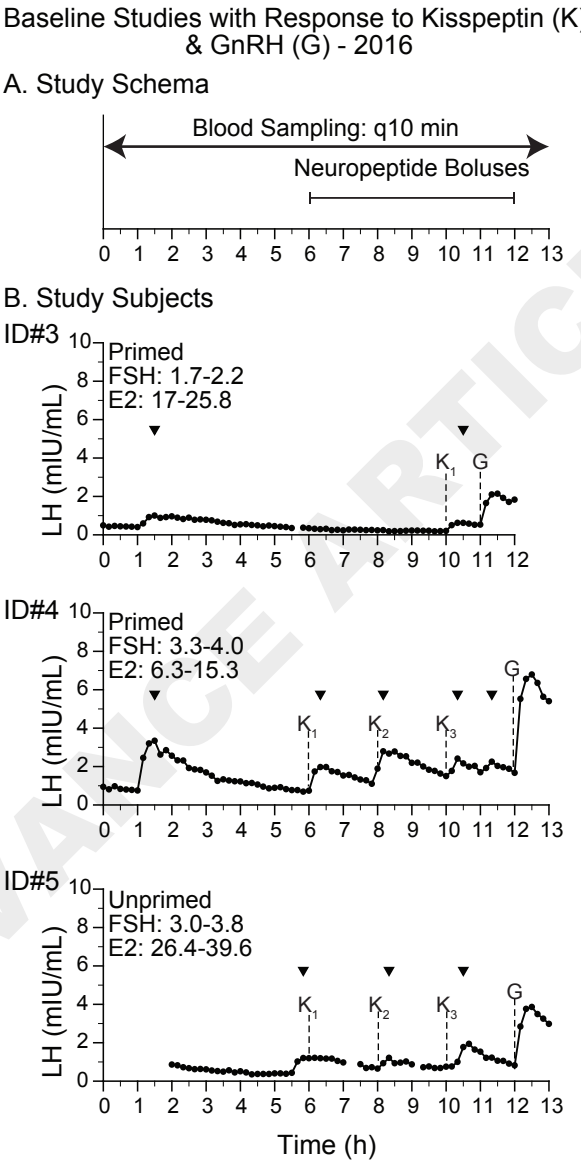
Table 1: Study Subject Characteristics

ID	Presentation	Initial Treatment and Subsequent Course	Research Study 2016				
			Protocol	FSH (IU/L)	LH (IU/L)	E_2 (pg/mL)	Imaging
TAC3 c.61_61delG p.A21LfsX44 heterozygote							
1	12.6y, menarche	12.6y – 35y, regular monthly menses	1) Baseline	4.23	2.18	20.4	US: endometrium 6mm, multiple small follicles
		35y, pregnant	2) IVB Kiss, GnRH				
TAC3 c.61_61delG p.A21LfsX44 homozygote							
2	15y, 1° amenorrhea, minimal thelarche	15-20y, HRT with breast development, growth spurt	Not applicable				
		20y, MPA X 10d +withdrawal bleed					
		mid-20s, HRT x 6mo					
		mid-20s, herbal medication					
		31y- present, amenorrheic					
3	14y, 1° amenorrhea, no thelache	16y8mo, FSH 2.1 IU/L (0.6-11), LH 1.1 IU/L (1-11), E_2 <40 pmol/L	1) Baseline & IVB Kiss, GnRH	2.12	0.49	20.3	normal MRI
		16-20y, HRT with breast development, growth spurt HRT	2) Kisspeptin Infusion & IVB GnRH				US – endometrium 4mm, all follicles <2 mm, uterus small adult size
		22y, FSH 7.7 IU/L (0.6-11), LH 11.9 IU/L (1-11)					
		22y, MPA x1 +withdrawal bleed					
		22y, spontaneous conception of healthy son, 1 most MPA					
		24y, superovulation x2 (MPA followed by CC), no pregnancies					
		24- 37y, ~ q3 mo MPA, + intermittent withdrawal bleeds					
		37-40y, amenorrheic					
		40y - present, restarted on ~ q3 mo MPA					
4	14y, 1° amenorrhea,	16y4mo, FSH 2.4 IU/L (0.6-11), LH <0.5 IU/L (1-11), E_2 49 pmol/L	1) Baseline & IVB Kiss,	3.97	0.94	11.2	US – endometrium 5mm, one follicle

	no thelache		GnRH				10mm, uterus small adult size
		16-22y, HRT with breast development	2) Naloxone Infusion & IVB Kiss, GnRH				
		22-24y, amenorrheic					
		24y,+ home pregnancy test followed by SAB					
		24y, FSH 5.5 IU/L (0.6-11), LH 5.4 IU/L (1-11)					
		25-27y, HRT					
		28y5mo, herbal medication, 2 spontaneous cycles 6 mo apart					
		28y, FSH, LH "normal range", E_2 "low" at 52 pmol/L					
		29 -30y, HRT					
		30-31y, amenorrheic					
		31- present, intermittent HRT use					
5	13y, 1° amenorrhea, no thelarche	17-18y, HRT	1) Baseline & IVB Kiss, GnRH	3.42	0.86	34	normal MRI
		21y, OCPs for 6 mo	2) Naloxone Infusion & IVB Kiss, GnRH				US – endometrium 9mm, cyst 3cm
		25y, herbal medication +withdrawal bleed, repeated without effect					
		26-28y, amenorrheic					
		28-29y, regular monthly cycling (1.3 y)					
		29-31y, q2.5 mo cycles (2.5y)					
		31y-present, yearly spontaneous spotting					

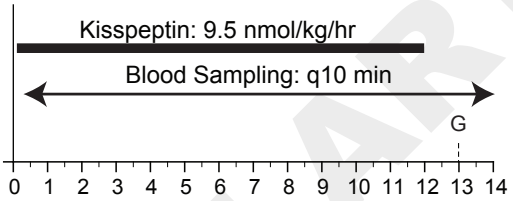
FSH = follicle stimulating hormone, LH = luteinizing hormone, E_2 = estradiol, IVB = intravenous bolus, kiss = kisspeptin, GnRH = gonadotropin stimulating hormone, US = transvaginal ultrasound, HRT = hormone replacement therapy, MPA = medroxyprogesterone acetate, CC = clomiphene citrate, SAB = spontaneous abortion, OCPs = oral contraceptive pills





Response to Kisspeptin Infusion & GnRH (G)

A. Study Schema



B. Study Subject ID#3