NKB Signaling in the Medial Amygdala Stimulates Gonadotropin Release in a Kisspeptin-
Independent Manner in Female Mice.

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Abstract

Neurokinin B (NKB) signaling is critical for reproduction in all studied species. The existing consensus is that NKB induces GnRH release via kisspeptin (Kiss1) stimulation in the arcuate nucleus. However, the stimulatory action of NKB is dependent on circulating estrogen (E2) levels, without which, NKB inhibits LH release. Importantly, the evidence supporting the kisspeptin-dependent role of NKB, derives from models of persistent hypogonadal state [e.g. Kiss1r knock-out (KO) mice], with reduced E2 levels. Here, we demonstrate that in the presence of E2, NKB signaling induces LH release in a kisspeptin-independent manner. Moreover, senktide (NKB receptor agonist) delivery to the medial amygdala (MeA) increases LH in E2-treated Kiss1 KO females (but not males or sham-treated females) similar to controls, and thus, this increase is independent of Kiss1 neurons. These results document a novel kisspeptin-independent regulatory pathway of reproductive function in females mediated by NKB-responsive neurons in the MeA.


**Introduction**

Reproduction is regulated by a complex neuronal network the precise components of which are still being elucidated. Nonetheless, it is well established that kisspeptin and neurokinin B (NKB) signaling systems are indispensable parts of this network. Loss-of-function mutations in the genes encoding for kisspeptin (*Kiss1*) or its receptor (*Kiss1r*) or NKB (encoded by the *Tac2* gene) and its receptor (NK3R, encoded by the *Tacr3* gene) in humans and mice, are linked to hypogonadotropic-hypogonadism and infertility (Seminara et al., 2003, Topaloglu et al., 2009).

Kisspeptins, secreted from Kiss1 neurons in the arcuate (*Kiss1*\(^{ARC}\)) and anteroventral periventricular/periventricular (*Kiss1*\(^{AVP/PeN}\)) nuclei, have been directly linked to the release of gonadotropin releasing hormone [GnRH (Smith et al., 2006, Fergani and Navarro, 2016)] however, there is another population of Kiss1 neurons in the medial amygdala (*Kiss1*\(^{MeA}\)) whose function has not yet been elucidated (Fergani and Navarro, 2016, Smith et al., 2006, Pineda et al., 2017). In addition to kisspeptin, *Kiss1*\(^{ARC}\) neurons express neurokinin B and dynorphin and these neurons are sometimes referred to as KNDy neurons (Fergani and Navarro, 2016). Functional studies in mice and other species suggest that the stimulatory effect of NKB/NK3R signaling lies up-stream of Kiss1/Kiss1r, which in turn, directly activates GnRH neurons and, hence, stimulates luteinizing hormone (LH) and follicle stimulating hormone (FSH) secretion into the peripheral circulation (Fergani and Navarro, 2016). Specifically, NKB signaling onto kisspeptin occurs via the auto-synaptic activation of NK3R residing on *Kiss1*\(^{ARC}\) neurons (Fergani and Navarro, 2016). The existence of this pathway is supported by the fact that the selective NK3R agonist, senktide, induces Fos expression in *Kiss1*\(^{ARC}\) neurons in vivo (Navarro et al., 2011a) and increases of their electrical activity in hypothalamic slices (de Croft et al., 2013, Navarro et al., 2011b). Subsequently, kisspeptin signaling was deemed an indispensable part of the reproductive role of NKB, as the stimulatory effect of senktide on LH secretion was shown to be absent in *Kiss1r* knock out (KO) mice (Garcia-Galiano et al., 2012, Navarro et al., 2015), prepubertal rats
treated with a Kiss1 antagonist (Grachev et al., 2012) or agonal juvenile monkeys with a desensitized Kiss1 (Ramaswamy et al., 2011). These studies clearly indicated the importance of NKB signaling onto Kiss1<sup>ARC</sup> neurons for GnRH/LH secretion.

Interestingly, the effect of NK3R activation via intracerebroventricular (ICV) administration of senktide on LH release is highly dependent on the sex steroid milieu; senktide was inhibitory in the absence but stimulatory in the presence of sex steroids in mice (Navarro et al., 2015) and sheep (Billings et al., 2010). This poses a predicament due to the fact that the aforementioned studies, rendering kisspeptin signaling indispensable for NKB stimulation of LH, have all been carried out in animal models characterized by a persistent hypogonadal state and therefore, in the absence of sex steroids. Furthermore, a subset of GnRH neurons have been shown to contain NK3R in rats (Krajewski et al., 2005) and mice (Navarro et al., 2015), and a kisspeptin-independent activation of GnRH neurons by NK3R agonists in the median eminence (ME) has been demonstrated <i>in vitro</i> (Gaskins et al., 2013). Thus, additional regulation of GnRH release at a different level, i.e. kisspeptin-independent action, by NKB in the presence of sex steroids, cannot be excluded.

The experimental studies described here aimed to assess whether senktide can stimulate LH release, in the presence of E<sub>2</sub>, in adult mice that lack a functional kisspeptin signaling system, and if so, investigate the potential mechanisms involved. We used mice in which Cre recombinase was targeted to the <i>Kiss1</i> locus and prevented Kiss1 protein synthesis; consequently, homozygous mice (<i>Kiss1<sup>cre/cre</sup></i>) are Kiss1 KO and display severe hypogonadotropic hypogonadism (Padilla et al., 2018). Our findings reveal a novel kisspeptin-independent pathway of GnRH/LH release in the female mouse, which is activated in the presence of E<sub>2</sub> and involves NKB/NK3R signaling within the medial amygdala (MeA).
Results

Central (ICV) administration of senktide stimulates LH release in female Kiss1 KO mice in the presence of sex steroids.

To investigate potential kisspeptin-independent stimulation of LH after central activation of NK3R signaling with senktide, we compared hypogonadal Kiss1 KO mice (Padilla et al., 2018) of both sexes to gonadectomized [GNX; orchidectomy (WTORX) or ovariectomy (WTOVX)] adult WT male and female mice. In the absence of sex steroids [testosterone (T) or E2, in males and females, respectively]] ICV senktide administration decreased plasma LH levels by ~50% in WT males ($P<0.0001$) and ~36% in WT females ($P=0.0016$) with no alteration observed in Kiss1 KO mice of either sex (Figure 1A, B). When circulating levels of sex steroids were restored, LH release was significantly increased in WT males and females [(WTOVX+E2, WTORX+T); $P=0.007$ and $P=0.0049$, respectively; Figure 1C, D]. Interestingly, the same was observed in Kiss1 KO female mice supplemented with E2 [(Kiss1 KO+E2); $P=0.005$; Figure 1D] but not in male Kiss1 KO mice supplemented with T (Kiss1 KO+T; Figure 1C) revealing the existence of a female-specific, kisspeptin-independent but E2-dependent, NKB/NK3R signaling pathway that controls LH release.

Chemogenetic activation of the ARC KNDy neuron stimulates LH release in control but not Kiss1 KO female mice.

A Cre-dependent activating DREADD (hM3D-Gq) tagged with mCherry and packaged in an adeno-associated virus (serotype 5; Figure 2A) was injected into the ARC of Kiss1Cre/+ or Kiss1 KO (i.e. Kiss1Cre/Cre) mice. Analysis following the completion of pharmacological studies demonstrated that mCherry expression was present throughout the ARC (Figure 2B) and not elsewhere. There was limited variability in the spread of mCherry among animals which extended throughout the medial-caudal extent of the ARC (approximately -1.40 mm to -2.30 mm from bregma; paxinos atlas). Six out of twenty mice (distributed among the groups) had primarily
unilateral m-Cherry spread, but they did not differ significantly from their respective groups in LH concentrations, and were therefore, included in further analyses. Within the ARC, of both Kiss1<sup>Cre/+</sup> and Kiss1 <sup>KO</sup> genotypes, HM3D:mCherry was expressed in ~32 % of GFP-immunoreactive cells (expressed in Kiss1 neurons in this mouse model), and was not observed in non-GFP cells or other brain areas.

Kiss1<sup>Cre/+</sup> animals treated with clozapine N-oxide (CNO) showed a significant increase in LH within the first 15 min after the injection (\(P=0.0057\)) compared to controls, which was sustained until the end of the sampling period (90 min; \(P<0.0001\); Figure 2C). However, no effect on LH release was observed in E2-supplemented Kiss1 KO (i.e. Kiss1<sup>cre/cre</sup>) animals treated with either saline or CNO (Figure 2D). Therefore, only mice with an intact Kiss1 signaling system showed an increase in LH release, despite similar activation of the Kiss1<sup>ARC</sup> neuron in both mouse models after CNO treatment. Thus, the stimulation of LH release after senktide in Kiss1 KO animals must occur via a different NK3R-expressing neuronal population.

**Senktide administration into the MeA, but not the ARC or POA, stimulates LH release in female WT and Kiss1 KO mice, in the presence of estrogen.**

The distribution of NK3R was investigated in WT and Kiss1 KO mouse brain sections (Figure 3, 4 and Supplemental Figure 4 and 5). Large NK3R immunoreactive neurons and fibers were identified in the substantia innominata, paraventricular nucleus, supraoptic nucleus, lateral hypothalamus, zona incerta, and perifornical regions. Interestingly, in the ARC, cells containing NK3R were evident only in WT<sub>ovx</sub> and hypogonadal Kiss1 KO female mice (Figure 3A and Supplemental Figure 4), whereas, in the MeA, NK3R cell bodies appeared only when WT and Kiss1 KO females were supplemented with E2 (Figure 4A, 4B and 4C).

Next, we investigated the anatomical relationship of NK3R and GnRH expression with dual-label immunohistochemistry, which revealed close appositions between the two proteins in the ARC and medial septum (MS) (Figure 3A, 3C). In these two areas, GnRH cells and fibers
showed dense intermingling and multiple foci of close apposition with NK3R containing cells and fibers, but no co-expression within GnRH cell bodies (>100 cells analyzed from a total of 16 mice; Figure 3C). Interestingly, we observed no fibers containing NK3R immunoreactivity in the internal or external zone of the median eminence (ME; Figure 3A, 3B).

To identify the brain area in which NK3R receptive neurons that mediate the kisspeptin-independent GnRH release reside, we stereotaxically administered senktide specifically into the ARC, the POA (at the level of the MS) or the MeA. These areas are prime candidates to play a role in LH stimulation because (a) they contain NK3R, the immunoreactivity of which is regulated by E₂, (b) there is an anatomical overlap of NK3R and GnRH protein, at least in the MS and ARC and (c) they contain GnRH and/or Kiss1 cell bodies and fibers and are known to play an important role in reproductive function (Smith et al., 2006, Kim et al., 2011). Senktide administration into the ARC or POA of WT_0X+E₂ mice stimulated LH secretion within 15 min from drug infusion (Figure 3D, 3E; P<0.0001 for both) compared to Kiss1 KO animals. However, when senktide was administered into the MeA of WT_0X+E₂ and Kiss1 KO females supplemented with E₂ a robust increase in LH was observed within 15 min after senktide infusion that was similar in both genotypes (Figure 4D). Conversely, in the absence of E₂, Kiss1 KO females did not show any alteration in LH release (Figure 4D), mimicking the LH responses we obtained after an ICV injection of senktide in these animals (Figure 1B).

Chemogenetic activation of the MeA Kiss1 neuron stimulates LH release in WT but not Kiss1 KO female mice.

Similar to the experiments described above, we delivered Cre-dependent AAV5-DIO-hM3Dq:mCherry to the MeA of Kiss1^{Cre/+} or Kiss1^{Cre/Cre} mice. HM3Dq:mCherry expression was present in the MeA (Figure 4F) and was limited to sections ranging from (from -1.6 mm to -2.0 mm from bregma; paxinos atlas). Two out of eight mice (one from each group) had primarily unilateral spread of the DREADD, but they did not differ significantly from their respective groups.
in LH concentrations, and were therefore, included in further analyses. Within the MeA, mCherry cell bodies were co-expressed in ~89 % of GFP-immunoreactive cells (i.e. Kiss1 cells), and was not observed in non-GFP cells.

*Kiss1<sup>Cre<sup>+</sup></sup> mice expressing hM3Dq:mCherry in the MeA and treated with CNO to activate the Kiss1<sup>MeA</sup> neurons, had an increase in LH within 30 min after the injection (*P*=0.0107) compared to animals receiving saline treatment, which was sustained for another 30 min before returning to basal levels (Figure 4G). No alteration in LH was observed in E<sub>2</sub>-supplemented *Kiss1* KO animals treated with either saline or CNO (Figure 4H).

**Discussion**

Our results provide evidence that the MeA is a component of the gonadotropin axis. Specifically, we have identified two independent pathways within the MeA that can lead to the stimulation of GnRH/LH release. The first involves Kiss1<sup>MeA</sup> neurons, the activation of which, stimulates LH release into the peripheral circulation. Furthermore, this is achieved by the release of kisspeptin and not by any other signaling molecules produced within the Kiss1<sup>MeA</sup> neuron, since LH was increased only in animals with an intact kisspeptin signaling system. A second pathway, involving NKB/NK3R signaling, was also identified, when senktide (NK3R agonist) administration into the MeA induced LH release in *Kiss1* KO mice (Padilla et al., 2018). Thus, kisspeptin is not a required mediator between NK3R activation in the MeA and LH secretion. Interestingly, this pathway is female-specific and estrogen-dependent as responses were absent in males and hypogonadal females.

From a mechanistic point of view, the most likely kisspeptin-independent pathway for LH stimulation by NKB would involve the direct regulation of GnRH release (Krajewski et al., 2005). Despite there being an anatomical overlap of GnRH and NK3R protein, specifically in the ARC and POA (at the level of the MS), we observed no instances of colocalization between NK3R
and GnRH cell bodies irrespective of the presence or absence of sex steroids. This reveals certain anatomical differences to what has been previously demonstrated in the rat, where ~16% of GnRH cell bodies were found to contain NK3R protein (Krajewski et al., 2005).

However, our results agree with reports of no NK3R expression in GnRH neurons of the ewe (Amstalden et al., 2010) suggesting the existence of species differences. Overall, our data suggest that the kisspeptin-independent action of NKB cannot be attributed to direct stimulation of NK3R located on GnRH neurons.

Kiss1^ARC (KNDy) neurons make close appositions with GnRH cell bodies and terminals (Lehman et al., 2010) and may therefore stimulate GnRH neurons through intermediates other than kisspeptin. For example, it has been demonstrated with in vitro examination of coronal brain slices, that senktide induces GnRH release from the ME and this effect is, in part, present in Kiss1 KO mice (Gaskins et al., 2013). We did not observe any NK3R immunoreactive fibers in the internal or external zone of the median eminence, indicating a potential lack of direct NKB (or senktide) regulation of the GnRH terminals in that area. Nonetheless, other signaling molecules such as glutamate (Nestor et al., 2016) or galanin, γ-aminobutiric acid (Skrapits et al., 2015) can potentially stimulate LH secretion and must also be considered. However, activation of the Kiss1^ARC (KNDy) neuron stimulated LH release only in mice with an intact kisspeptin signaling system and was completely absent in Kiss1 KO mice. This provides evidence that kisspeptin, but no other signaling molecule produced by Kiss1^ARC (KNDy) neuron can stimulate LH release in vivo.

The distribution of NK3R has been described in the human, rat, and ewe (Mileusnic et al., 1999, Krajewski et al., 2005, Amstalden et al., 2010) and here, we confirm a similar distribution in the mouse brain. Interestingly, in certain areas the immunoreactivity of NK3R-containing cell bodies was highly dependent on sex steroid levels. Specifically, estrogen downregulated NK3R expression in the ARC whereas the opposite was true for the MeA, with more NK3R containing...
cell bodies evident when animals were supplemented with E2. High sensitivity of NK3R expression
to E2, has also been reported for the ARC with *in situ* hybridization studies (Navarro et al., 2009).
Interestingly, this regulation of NK3R expression is reminiscent of the regulation of *Kiss1* by E2 in
these areas (Kim et al., 2011, Smith et al., 2006).

Based on the aforementioned anatomical observations we proceeded with senktide
administration into the ARC, POA (at the level of the MS) and MeA of E2-treated animals in order
to locate the kisspeptin-independent, LH-stimulating population of NK3R-expressing neurons.
Senktide administration into the ARC or POA (at the level of the MS) significantly stimulated LH
secretion in WT females compared to *Kiss1* KO animals. Therefore, our data suggest that LH
release, as a result of NKB/NK3R signaling in the POA or ARC, is predominantly achieved via
initial kisspeptin release, involving *Kiss1*AVPV/PeN and/or *Kiss1*ARC neuron activation. Indeed, 10%
of *Kiss1*AVPV/PeN and virtually all *Kiss1*ARC neurons contain NK3R (Navarro et al., 2015). Moreover,
both populations are interconnected, *Kiss1*ARC cells project to *Kiss1*AVPV/PeN neurons and GnRH
cell bodies and terminals (Yip et al., 2015), which could also account for the increase in LH after
administration of senktide into the POA. However, a slight increase in LH occurred in the absence
of kisspeptin signaling (*Kiss1* KO mice). In the ARC and MS of the POA, we observed several
instances were NK3R containing fibers were in close apposition to GnRH cell bodies and/or
processes indicating a potential presynaptic action of NKB onto GnRH. It is possible that NKB (or
senktide) signaling onto presynaptic NK3R, results in the enhanced secretion of other stimulatory
neuropeptides, which in turn stimulate GnRH secretion and could account for the slight increase
in LH observed in *Kiss1* KO mice, as recently documented in the rat striatum, in which tachykinins
(including NKB) presynaptically stimulate the release of dopamine (Glowinski et al., 1993).

Interestingly, senktide administration into the MeA of females supplemented with E2
produced a similar robust increase in LH in animals with or without the presence of the *Kiss1*
gene. Conversely, hypogonadal *Kiss1* KO females did not show any alteration in LH release,
indicating that this kisspeptin-independent NKB/NK3R signaling mechanism in the MeA becomes activated only when E$_2$ is present. This notion is further supported by our finding that the number of NK3R cells increases with E$_2$, and this upregulation is specific to the MeA. NK3R$_{MeA}$ expressing cells do not co-localize with NKB, but are surrounded by a plethora of NKB fibers (Supplemental Figure 5) the source of which remains to be determined. Likely candidates include NKB-expressing cells residing within the ARC or the neighboring NKB population in the central amygdala (CeA) (Supplemental Figure 5) to date, implicated only in the modulation of fear memories (Andero et al., 2016).

To investigate the mechanism further, we determined whether this is mediated directly or indirectly by Kiss1$_{MeA}$ neurons through the release of kisspeptin or other signaling molecules. Chemogenetic activation of the Kiss1$_{MeA}$ neuron provided evidence that the activation of Kiss1 neurons in the MeA of the female mouse can stimulate LH but only through the release of kisspeptin. This, clearly demonstrates the influence of Kiss1$_{MeA}$ signalling on the gonadotropic axis. Furthermore, GnRH/LH stimulation via the Kiss1$_{MeA}$ neuron is not part of the kisspeptin-independent NKB/NK3R signaling pathway but of a second LH stimulating mechanism originating from the MeA. To date, functional studies in rodents have shown that an injection of kisspeptin specifically in to the amygdala results in increased LH secretion, while blocking endogenous amygdala kisspeptin signalling with a kisspeptin antagonist decreases both LH secretion and LH pulsatility (Comninios et al., 2016) indicating that the kisspeptin released may act locally, within the MeA, as part of the LH stimulating pathway.

The functional relevance of either the MeA kisspeptin-dependent or the kisspeptin-independent neuronal population that can induce LH release is unknown. A reasonable hypothesis is that Kiss1$_{MeA}$ and NK3R$_{MeA}$ neurons are part of the neuronal network linking pheromonal/social cues and gonadotropin release (Yang et al., 2018). Indeed, estrogen receptors are expressed in the MeA (Lymer et al., 2018) and the brain region is a central hub for processing
sensory inputs such as olfactory signals and integrating these into behavioral (Rajendren and Moss, 1993, Adekunbi et al., 2018) and neuroendocrine outputs (Pineda et al., 2017). Specifically, it is compelling to hypothesize that the kisspeptin-independent, NK3R-dependent pathway is employed for the generation and/or enhancement of the LH surge and/or female sexual behavior, e.g. lordosis, given that this mechanism was absent in male mice, and is exclusively activated in the presence of estrogen, similar to what is observed in the female AVPV/PeN (Smith et al., 2006).

In accordance, recent evidence demonstrated the enhancement of the LH surge in rats exposed to male-soiled bedding, which was accompanied by an increased Fos expression in Kiss1AVPV/PeN neurons as well as various limbic structures, including the MeA (Watanabe et al., 2017, Hellier et al., 2018).

In summary, we have shown that the MeA is a previously unknown component of the gonadotropic axis. Initially, we observed that senktide administration into the lateral ventricle stimulates LH release into the peripheral circulation of female mice lacking kisspeptin (Kiss1 KO), but only when they are supplemented with E2. Upon further investigation, we identified two mechanisms that can lead to GnRH/LH secretion in the female, involving Kiss1MeA or NK3R-expressing neurons located in the MeA. Collectively, these data demonstrate that the gonadotropic axis is subject to regulation by signalling originating outside the hypothalamus and specifically the MeA, involved in the regulation of social behaviors including sexual behavior, anxiety, and olfaction.

Methods

Animals

A Kiss1Cre:GFP knock-in mouse (version 2) was generated from C57Bl/6 blastocysts and verified at the University of Washington (Padilla et al., 2018). The homozygous version of this mouse, Kiss1Cre:Cre is a Kiss1 KO as characterized elsewhere (Padilla et al., 2018). Kiss1 gene was also confirmed undetectable from POA and MBH tissue (Supplement Figure 1). Animals
were group housed according to sex and bred under constant conditions of temperature (22–
24°C) and light [12 h light (06:00)/dark (18:00) cycle], fed with standard mouse chow (Teklad F6
Rodent Diet 8664) and were given \textit{ad libitum} access to tap water. For all studies, C57Bl/6 WT or
\textit{Kiss1}^{Cre/+} (heterozygous state) males or females between age 8 and 20 weeks were used and
studied in parallel to \textit{Kiss1}^{Cre/Cre} (\textit{Kiss 1} knock-out state) littermates. In order to test the specificity
of the NK3R antibody, NK3RKO mice were used as described below.

**Experiment 1: Effect of central (ICV) administration of senktide on LH release in male and
female WT and Kiss1 KO mice with or without the presence of sex steroids.**

In this experiment we aimed to assess whether central activation of NK3R signaling with
senktide (an NK3R specific agonist), can stimulate LH release in \textit{Kiss1} KO mice (i.e., in a
kisspeptin-independent manner) in the presence or absence of sex steroids. Adult WT male and
female mice were GND and studied in parallel to hypogonadal (with low sex steroid levels) \textit{Kiss1}
KO littermates (n=10/group). ICV injections (see below) of senktide (Tocris Biosience, Cat. No.
1068; 600 pmol diluted in 5µl 0.9% NaCl) were performed and blood samples were collected
before (basal) and 25 min after ICV injection for LH measurements as has been previously
described (Navarro et al., 2015). Next, animals were implanted with sex steroids (n=10/group)
and the ICV experiment was repeated a week later. The dose of senktide used, and the time of
blood collection were selected based on our previous studies (Navarro et al., 2015).

**Experiment 2: Effect of ARC KNDy neuron chemogenetic activation on LH release in WT
and Kiss1 KO female mice in the presence of estradiol.**

In order to determine whether the release of other components, besides kisspeptin, within
the \textit{Kiss1}^{ARC} (KNDy) neuron can stimulate LH release, we used a chemogenetic approach to
specifically activate \textit{Kiss1}^{ARC} neurons of \textit{Kiss1}^{Cre/+} or \textit{Kiss1}^{Cre/Cre} mice treated with \textit{E}_{2} (n=5-8/group). Females received bilateral stereotaxic injections (see below) of an adeno-associated
virus (pAAV) encoding a Cre-driven Gq-coupled DREADD (pAAV5/hSyn-DIO-hm3Dq:mCherry;
Addgene, Cat. No.44361-AAV5; titer $3 \times 10^{12}$ genome copies per ml; 1 µl per hemisphere). Following infection, mice were given 3 weeks for recovery and maximum expression of the AAV vector. On the day 1 of the experiment animals were administered an ip bolus injection of vehicle saline (0.9% NaCl; day 1) and then hM3D receptors were activated by ip injection of its agonist, clozapine N-oxide (CNO; 10 mg/kg dissolved in saline; day 2). Blood samples were collected just before saline or CNO treatment (0) and then every 15 min for 90 min. The dose of CNO was chosen based on previous behavioral studies using hM3Dq manipulations (Ben-Shaanan et al., 2016). At the end of the experiment, all mice were treated with an icv injection of senktide, as a control, and to confirm that animals were appropriately treated and primed.

**Experiment 3: Effect of senktide administration in to the ARC, POA or MeA on LH release in female WT and Kiss1 KO mice with the presence of estrogen.**

In this set of experiments, we aimed to locate the brain area which senktide is acting to stimulate LH release. To this end, we first conducted neuroanatomical studies to confirm NK3R protein expression in the mouse hypothalamus, as well as to investigate the potential anatomical interplay between NK3R and GnRH neurons, as the most plausible kisspeptin-independent mechanism. Thus, WT\textsubscript{OVX}, WT\textsubscript{OVX+E2}, Kiss1 KO and Kiss1 KO+E2 (n=5/group) were perfused following standard protocols and the brains were collected for immunohistochemical (IHC) analyses, as described in detail below. Based on IHC results, a stereotaxic injection approach was used to specifically activate NK3R in the POA (at the level of the MS), or ARC or MeA and monitor LH responses in the peripheral circulation of anesthetized WT\textsubscript{OVX+E2} and Kiss1 KO+E2 females. Unilateral injections were performed as described below on WT\textsubscript{OVX+E2}, and Kiss1 KO+E2 (n=5/group) which received 600 pmol of senktide diluted in 1µl saline (0.9% NaCl) in the POA or ARC or MeA. An additional control group was added to the MeA injected cohort which consisted of Kiss1 KO mice without E\textsubscript{2} treatment (n=5), which according to results from experiment 1 should not lead to an increase in LH after senktide administration. Blood samples were collected before
and then every 15 min for 45 min after senktide administration. The first blood sample (15 min post administration) was taken with the needle still in place. Lastly, animals were decapitated and the brains collected, frozen on dry ice and stored at -80°C for injection site confirmation (see below).

**Experiment 4: Effect of MeA Kiss1 neuron chemogenetic activation on LH release in WT and Kiss1 KO female mice in the presence of estradiol.**

To determine whether the release of other components, besides kisspeptin, within the Kiss1MeA neuron can stimulate LH release, we used a similar approach as previously described to specifically activate Kiss1MeA neurons of Kiss1Cre/+ or Kiss1Cre/Cre mice treated with E2 (n=5/group). E2 is known to upregulate Kiss1 expression in the MeA and Cre:GFP expression follows an identical pattern in this mouse model (Padilla et al., 2018). Thus, hypogonadal Kiss1Cre/Cre mice injected with the Cre-dependnet hM3Dq:mCherry in the MeA were also treated with an E2 capsule prior to surgery. The remainder of the experimental protocol was similar to what has been described in experiment 2.

**Gonadectomy and sex steroid replacement**

The effects of sex steroids or lack thereof on LH secretion was established via bilateral GND; (Strom et al., 2012, Idris, 2012]) of adult male and female WT mice (WTORX and WTOVX) with circulating sex steroid levels being restored between genotypes (WTORX+T, WTOVX+E2, Kiss1 KO+T and Kiss1 KO+E2) via subcutaneous implantation of capsules (1.5 cm long, 0.078 in inner diameter, 0.125 in outer diameter; Dow Corning) containing 50µg/ml 17β-estradiol (Sigma-Aldrich), in sesame oil or testosterone in powder form (1 cm filled area). Neuroendocrine experiments were consistently conducted 7 d after gonadectomy or sex steroid supplementation (Garcia-Galiano et al., 2012).

**Kiss1 KO GnRH priming**

To exclude the possibility that absence in gonadotropin responses to the various stimuli in
hypogonadal Kiss1 KO mice may result from inadequate pituitary responsiveness to GnRH, which has been previously described in animals with a defective Kiss1 signaling system (Roa et al., 2008) Kiss1 KO mice were subjected to a protocol of GnRH priming during 2 days prior testing, as has been previously described (Garcia-Galiano et al., 2012). In this protocol, each mouse received five successive ip boluses of a low dose of GnRH (0.15 µg/each), with the following schedule: at 10:00 h, 17:00 h, and 23:50 h on the first day; at 0800 and 1600 on the second day with neuroendocrine tests being conducted on the third day (Garcia-Galiano et al., 2012). WT mice injected with saline vehicle, following the same protocol, served as controls.

**Intracerebroventricular (ICV) Injections**

ICV injections were performed following previously published procedures (Navarro et al., 2015). Briefly, 2-3 days before the experiment, mice were anesthetized with isoflurane and a small hole was bored in the skull 1 mm lateral and 0.5 mm posterior to bregma with a Hamilton syringe attached to a 27-gauge needle fitted with polyethylene tubing, leaving 2.0 mm of the needle tip exposed. Once the initial hole was made, all subsequent injections were made at the same site. On the day of ICV injection experiments, mice were anesthetized with isoflurane for a total of 5-10 min, during which time 5 µl of solution were slowly and continuously injected into the lateral ventricle. The needle remained inserted for approximately 30 sec after the injection to minimize backflow up the needle track. Mice typically recovered from the anesthesia within 3 min after the injection.

**Stereotaxic injections**

Mice were deeply anaesthetized with isoflurane and placed into a stereotaxic apparatus (Kopf Instruments, Model 940). After exposing the skull via incision, a small hole was drilled for injection at the appropriate AP and ML coordinates. A syringe (Hamilton, 5 µL, Model 175 RN SYR, 32 ga, Cat. No.80016) was lowered into the brain at the appropriate DV coordinates. Coordinates relative to bregma were as follows: anteroposterior (AP) -1.6 mm, mediolateral (ML)
± 0.25 mm and dorsoventral (DV) -5.85 mm for the ARC, AP +0.6 mm, ML ± 0.25 mm, DV -5.15 mm for the POA and AP -1.9 mm, ML ± 2.0 mm, DV -4.9 mm for the MeA. Injection sites were chosen based on the Paxinos Brain Atlas, and confirmed with India Ink (Fisher Scientific, Cat. No. NC9903975) trial injections. Each infusion was slowly delivered over 2 min (500 nl/min), the needle was left in place for an additional 5 min (for AAV injections) and 15 min (for senktide administrations) and then slowly withdrawn to minimize backflow. Animals received 0.3 mg/kg buprunex (subcutaneous) during surgery and 24 h later for analgesia and were allowed a 3-week recovery before onset of experiments.

**Blood Samples and LH measurements**

In all cases blood samples for LH measurements were obtained after a single excision of the tip of the tail. The tip was cleaned with saline and then massaged prior to taking a 4 µl blood sample from the tail tip with a pipette. Whole blood was immediately diluted in 116 µl of 0.05% PBST [phosphate buffer saline (Boston Bio Products, Cat. No. BM220) containing Tween-20 (Sigma, Cat. No. P2287), vortexed, and frozen on dry ice. Samples were stored at -80°C until analyzed with LH ELISA (Steyn et al., 2013).

**Immunohistochemistry**

*Tissue preparation*

Animals were terminally anesthetized with a ketamine/xylazine in saline (0.9% NaCl) cocktail and transcardially perfused with 0.1 M phosphate-buffer (0.1M PB) followed by 4% paraformaldehyde diluted in 0.1M PB (PFA; Electron Microscopy Sciences). Brains were removed, stored in the same fixative for 4 hours and then transferred into sucrose solution [Thermo Fisher Scientific; 20% sucrose in 0.1 M PB containing 0.01% sodium azide (Sigma-Aldrich)] at 4°C. After sucrose infiltration tissue was cut into 30 µm coronal sections using a freezing stage microtome (Fisher HM440E). The tissue sections were separated into two groups of three parallel series (90 µm apart). The first group consisted of sections extending from the
medial septal nucleus to the caudal part of the retrochiasmatic area (+1.0 mm to -1.0 mm relative to bregma; containing GnRH cell bodies and the RVP3V population of Kiss1 cells) and the second encompassing the ARC nucleus (from -1.0 till -2.8 mm relative to bregma; containing ARC and amygdala Kiss1 populations of cells). Sections were stored at -20°C in cryoprotectant [30% sucrose in 0.1 m PB containing 30% ethylene glycol (Thermo Fisher Scientific) and 0.01% sodium azide] until further processing.

**General procedures**

For all staining procedures detailed below, free-floating sections at room temperature and under gentle agitation were thoroughly washed in PBS, pH 7.4, between all incubations, and treated with 10% H$_2$O$_2$ (10 min; in PBS) and PBS* [1 h; PBS containing 0.1% bovine serum albumin (Thermo Fisher Scientific) and 0.4% Triton X-100 (Sigma-Aldrich)]. Brain sections were mounted onto Superfrost plus glass slides (Fisher Laboratories), air dried and cover-slipped with Vectashield HardSet Mounting Medium (Vector Laboratories). For immunodetection of the different proteins, sections of all animals were processed simultaneously. Secondary antibodies were tested for non-specific binding by primary antibody omission. mCherry immunostaining was compared and validated with the endogenous mCherry expressed by the viral construct in a separate series of sections. The anti-GnRH and anti-NKB antibodies produced staining patterns identical to those from several independent GnRH and NKB antibodies (Merlo et al., 2007, Krajewski et al., 2005) and consistent with those by in situ hybridization (Duarte et al., 2006). The specificity of the NK3R antibody was tested by staining brain tissue collected from NK3R KO mice (True et al., 2015) alongside experimental tissue. No NK3R staining was observed in NK3RKO animals (Supplemental Figure 2).

**mCherry and GFP.** Brains from Kiss$^{1\text{Cre/}}$ and Kiss$^{1\text{Cre/Cre}}$ mice (n=5/group) injected with AAV5-hSyn-DIO-hM3D(Gq)-mCherry were assessed for mCherry reporter expression to confirm bilateral infection of ARC or MeA GFP expressing Kiss1 neurons with the AVV carrying Cre-
dependent hM3D receptor. One series of free-floating sections containing ARC and amygdala from each animal was incubated overnight in blocking solution containing rat anti-mCherry primary antiserum, Alexa Fluor 594 conjugate (1:500; Thermo Fisher, Cat. No. M11240). The next morning sections were extensively washed in PBS and then incubated overnight in rabbit anti-GFP tag antibody (1:5,000; Thermo Fisher, Cat. No. A-6455) followed by goat-anti-rabbit DyLight 488 secondary antibody (1:200; Thermo Scientific, Cat. No. 35552).

**NK3R and GnRH.** Brains from WT and Kiss1 KO mice were processed for NK3R and GnRH in order to compare the protein distribution of NK3R in the two genotypes and investigate the potential colocalization with GnRH in the hypothalamus. Furthermore, we processed tissue sections from WT(OVX), WT(OVX+E2), Kiss1 KO and Kiss1 KO+E2 groups to determine whether E2 regulates protein expression of NK3R in the a) preoptic area/medial septum, where most of the GnRH cell bodies are located b) ARC where KNDy cells reside, and c) MeA where senktide induced LH release. Hence, a series of every third section, extending from the level of the optic chiasma to the mammillary nucleus, was processed for NK3R and GnRH using a modified protocol previously described (Goodman et al., 2007). Tissue sections were incubated sequentially with: 1) rabbit anti-NK3R (1:30,000; Novus Biologicals, Cat. No. NB300-102) for 17 hours, 2) biotinylated goat anti rabbit IgG (1:500; Vector Laboratories, Cat. No. BA1000), for 1 hour, 3) avidin and biotinylated horseradish peroxidase complex (Avidin-Biotin Complex; 1:500; Vector Laboratories, Cat. No. PK-6100) for 1 hour, 4) biotinylated tyramine (1:250; PerkinElmer, Cat. No. NEL700A001KT), containing 0.003% H₂O₂ for 10 minutes, and 5) DyLight 488 conjugated streptavidin (1:200; Thermo Fisher, Cat. No. 35552) for 30 minutes. Next, sections were incubated with rabbit anti-GnRH (1:1,000; Abcam; Cat. No. ab5617) for 17 hours. The next morning, sections were washed and incubated with goat anti-rabbit Alexa 555 (1:200; Thermo Fisher, Cat. No. A-21428) for 30 minutes. The specificity of the NK3R antibody we used in our anatomical studies (see below) was tested by staining brain tissue collected from transgenic mice.
with mutations in the NKB receptor and previously described and validated [NK3R-/- mice (True et al., 2015)]

NK3R and NKB. An additional series of sections containing MeA from WT(OVX+E2) mice was processed for staining with NK3R as described above. Sections were further incubated overnight with rabbit-anti Neurokinin B (1:1000; Novus Biologicals, Cat. No. NB300-201) and goat anti-rabbit Alexa 555 (1:200) for 30 minutes.

Microscopy and image analysis

Validation of senktide injection site.

The locations of POA, ARC and MeA injection sites were investigated in sections cut at 20 μm thickness using a cryostat (Fisher, HM505E). Every other section was collected around the injection site, mounted on microscope slides air-dried and cover slipped with Vectashield HardSet Mounting Medium (Vector Laboratories, Burlingame, CA). Only animals with accurate and restricted injection sites were included in the analysis.

Validation of chemogenetic activation of ARC KNDy or MeA Kiss1 neurons.

Sections from animals injected with AAV vectors encoding hM3Dq:mCherry in the ARC or MeA were examined, and the location of mCherry expression was confirmed in GFP positive neurons. In both cases, quantification of GFP and GFP/mCherry positive neurons in all areas was carried out in a subpopulation of animals (n=4/group) with images taken at x20 magnification from 2 representative sections per animal.

NK3R/GnRH anatomical relationship

The anatomical relationship between NK3R/GnRH (throughout the hypothalamus), mCherry/GnRH (in the ARC) and NK3R/NKB (in the MeA) was examined in sections 90 μm apart, from each mouse. In addition, comparisons of NK3R cell numbers between WT OVX, WT OVX+E2, Kiss1 KO and Kiss1 KO+E2 (n=5/group) were performed in six to eight sections at 20X
magnification containing ARC and MeA, to determine the effect of E2 on protein expression in these areas. Counts were averaged per mouse, per brain area. A digital camera (CoolSnap EZ, PhotometricsTM, Canada) attached to a microscope (Nikon Eclipse 90i), with the appropriate excitation for DyLight 488 (green fluorescence) and Alexa 555 (red fluorescence) and NIS-Elements Viewer AR 310 software was used to examine tissue sections and superimpose two images and determine putative colocalization or interactions. Montages of images and adjustments of brightness and contrast levels were made in Adobe Photoshop CS5.

Statistics

All data are presented as mean ± SEM. Single point comparisons (basal LH versus after ICV injection), were made using 2-tailed paired t tests. Repeated LH concentrations at multiple time-points and between treatments were compared using a 2-WAY ANOVA and a Fishers posthoc test when appropriate. Area under the curve was compared with 2-tailed student t tests. A P value less than 0.05 was considered significant. All analyses were performed with GraphPad Prism Software, Inc (San Diego, CA).

Study Approval

All animal care and experimental procedures were approved by the National Institute of Health, and Brigham and Women's Hospital Institutional Animal Care and Use Committee, protocol #05165. The Brigham and Women's Hospital is a registered research facility with the U.S. Department of Agriculture (#14-19), is accredited by the American Association for the Accreditation of Laboratory Animal Care and meets the National Institutes of Health standards as set forth in the Guide for the Care and Use of Laboratory Animals (DHHS Publication No. (NIH) 85-23 Revised 1985).
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Author contributions

CF and VMN conceived and designed the research. CF, SL and AMJV conducted experiments. SLP and RDP generated and provided the Kiss1Cre mice and validated the Kiss1KO model used in the study. CF, SL and VMN contributed to data analysis. CF and VMN wrote the manuscript, and all authors contributed to manuscript editing.

Figure Legends

Figure 1. LH levels in male (A and C) and female (B and D) adult WT and Kiss1KO mice, from blood samples collected before (basal) and 25 min after ICV injection of 600 pmol senktide (an NK3R-specific agonist). (A, B) LH levels in WT male and female mice GNX (WTORX and WTOVX, respectively) and studied in parallel to hypogonadal Kiss1KO littermates (n=10/group). (C, D) LH levels in WT and Kiss1KO male and female mice with restored levels of sex steroids (WTORX+T, WTOVX+E2, Kiss1KO+T, Kiss1KO+E2; n=10/group). Paired t-test ****P<0.0001, **P<0.007 compared to basal levels of LH. T=testosterone, E2=estradiol.

Figure 2. (A) Schematic representation of the site of injection of an AAV encoding a Cre-dependent hM3Dq DREADD tagged to mCherry. (B) Representative photomicrograph of a coronal brain section stained for GFP (green), mCherry (red) and merged GFP and mCherry immunoreactivity in the ARC of a Kiss1KO female mouse >3 weeks after hM3Dq:mCherry injection (Scale bar 50 µm). Mean ± SEM LH responses and area under the curve (AUC) to an injection of saline (grey line-empty bar) or CNO (green line-green bar) of hM3Dq-injected
**Kiss1**<sup>Cre<sup>+/</sup> (C) and **Kiss1**<sup>Cre/Cre</sup> (KO D) female mice (n=5-8/group). 3V: third ventricle. *P<0.035, **P<0.006, ***P<0.0001.

**Figure 3.** Representative photomicrographs depicting dual label detection of NK3R (green) and GnRH (red) in the ARC (A) and ME (B) of WTovx and Kiss1 KO animals. (A) Panels on the right are enlarged images (scale bar: 20 µm) from boxed areas on the left (scale bar: 100 µm) showing numerous close appositions (arrowheads) between NK3R and GnRH in the ARC. (B) Enlarged images of the boxed area from the Kiss1 KO animal in (A) showing intense GnRH but lack of NK3R staining in the ME. (C) Dual-label detection of NK3R (green) and GnRH (red) within the region of the MS of E<sub>2</sub>-supplemented WT and Kiss1 KO animals [WT<sub>OVX+E2</sub> and Kiss1 KO<sub>E2</sub>, respectively; scale bar: 50 µm]. Arrowheads indicate sites of close apposition. (D, E) Mean ± SEM LH responses and area under the curve (AUC) to an injection of senktide into the ARC (D) or POA (E) of WT<sub>OVX+E2</sub> (blue line-blue bar) and Kiss1 KO<sub>E2</sub> (green line-green bar) female mice (n=5/group). 3V: third ventricle, ARC: arcuate, ME: median eminence, MS: medial septum. **P<0.0015, ****P<0.0001.

**Figure 4.** (A) Representative photomicrographs depicting NK3R-immunoreactive cell bodies and fibers in the MeA of (A) WT<sub>OVX</sub> (left panel) and WT<sub>OVX+E2</sub> (right panel) or Kiss1 KO (left panel) and Kiss1 KO<sub>E2</sub> (right panel). (C) Mean ± SEM number of NK3R-immunoreactive cells per 30 µm section in the MeA of WT<sub>OVX</sub>, WT<sub>OVX+E2</sub>, Kiss1 KO and Kiss1 KO<sub>E2</sub> animals (6-8 sections from 5 animals/group). (E) Schematic representation of the site of injection of an AAV encoding a Cre-dependent hM3Dq:mCherry. (B) Representative photomicrograph of a coronal brain section stained for GFP (green), mCherry (red) and merged GFP and mCherry immunoreactivity in the MeA of a Kiss1 KO female mouse >3 weeks after hM3Dq:mCherry injection (Scale bar 50 µm). (G, H) Mean ± SEM LH responses and area under the curve (AUC) to an injection of saline (grey
line-empty bar) or CNO (green line-green bar) of hM3Dq DREADD injected Kiss1<sup>Cre/Cre</sup> (G) and Kiss1<sup>Cre/Cre</sup> (KO; H) female mice (n=5/group). opt: optic tract. *P<0.025, **P<0.0014.

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Supplemental Materials and Methods

Quantitative real-time RT PCR
We aimed to confirm the lack of Kiss1 expression in the POA and MBH of Kiss1 KO mice. WT (OVX; n=3) and Kiss1 KO (n=5) female mice were killed, brains were exposed and the POA and MBH was extracted and immediately frozen in dry ice and stored at −80 C. Total RNA from both areas was isolated using TRIzol reagent (Invitrogen) followed by chloroform/isopropanol extraction. RNA was quantified using NanoDrop 2000 spectrophotometer (Thermo Scientific) and one microgram of RNA was reverse transcribed using Superscript III cDNA synthesis kit (Invitrogen). Quantitative real-time PCR assays were performed in triplicates of each sample on an ABI Prism 7000 sequence detection system, and analyzed using ABI Prism 7000 SDS software (Applied Biosystems). The cycling conditions were as follows: 2 min incubation at 50°C, 10 min incubation at 95°C (hot start), 40 amplification cycles (95°C for 15 s, 60°C for 1 min, and 45 s at 75°C, with fluorescence detection at the end of cycles 3 – 40), 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. The primers used are listed in Table 1. The data were normalized using L19 primers as an internal control. Kiss1 expression was detected using primers: F- CTCTGTGTCGCCACCTATGC R – TTCCCAGGCATTAACGAGTTC. Values were normalized with housekeeping gene Rpl19.

Immunohistochemistry for mCherry and GnRH
mCherry and GnRH. Brains from Kiss1Cre/+ and Kiss1Cre/Cre mice (n=5/group) injected with AAV5-hSyn-DIO-hM3D(Gq)-mCherry were assessed for mCherry reporter expression and GnRH to confirm anatomical integrity of Kiss1 neuron and GnRH fiber interaction in the area. A potential explanation for the lack of LH responses in DREADD-injected Kiss1 KO mice could be developmental alterations in the projections from KNDy neurons to GnRH neurons 1. Furthermore, that could explain the lack of LH stimulation after chemogenetic activation of

1 Weller et al.
Kiss1 neurons in Kiss1 KO mice. The anatomical relationship between the two proteins appeared to be similar in both genotypes (Kiss1\textsuperscript{Cre+/+} and Kiss1 KO), suggesting that kisspeptin is not needed developmentally for the formation of fibers in Kiss1 neurons. (Supplemental Figure 3). One series of free-floating sections containing ARC and amygdala from each animal was incubated overnight in blocking solution containing rat anti-mCherry primary antiserum, Alexa Fluor 594 conjugate (1:500; Thermo Fisher, Cat. No. M11240). The next morning sections were extensively washed in PBS and then incubated overnight in rabbit anti-GnRH (1:1,000; Abcam, Cat. No. ab5617) followed by goat-anti-rabbit DyLight 488 secondary antibody (1:200; Thermo Scientific, Cat. No. 35552).

Supplemental Figure 1. Expression profile of A) Kiss1 gene in the mediobasal hypothalamus (MBH), B) Kiss1 gene in the preoptic area (POA), of ovariectomized (OVX) WT and hypogonadal Kiss1 KO female mice. Comparison between groups was carried out with a student’s t-test (\(* P < 0.001\)). The data were normalized using L19 primers as an internal control.

Supplemental Figure 2. NK3R antibody validation. Representative photomicrographs of sections processed for immunofluorescent detection of NK3R in NK3R KO female mice. The tissue was derived from females that were either OVX or OVX and E\(_2\)-treated known to induce maximal NK3R expression in the ARC and MeA, respectively. A) Complete absence of staining in the ARC of an OVX NK3R KO female mouse. B) Complete absence of staining in the MeA of an OVX and E\(_2\)-treated NK3R KO female mouse. Scale bar: 100 µm.

Supplemental Figure 3. Representative merged images of mCherry (red) and GnRH (green) immunoreactivity in the ARC of Kiss1\textsuperscript{Cre+/+} (A) and Kiss1\textsuperscript{CreCre} (B). Right panels are higher magnifications (Scale bar 20 µm) of boxed areas from left panels (Scale bar 50 µm).
Supplemental Figure 4. Representative photomicrographs depicting dual label detection of NK3R (green) and GnRH (red) in the ARC of WT(OVX+E2) and Kiss1 KO+E2 animals. Scale bar: 100 µm. Note the lack of NK3R staining as opposed to the absence of E2 in the ARC (Figure 3).

Supplemental Figure 5. Representative photomicrographs of a coronal section stained for NK3R (green), NKB (red) and merged NK3R and NKB immunoreactivity in the amygdala of (A) WT(OVX+E2) and NKB (green) of (B) WT(OVX) female mouse. Scale bar: 150 µm (C) Enlarged images depicting NKB fibers (presumably from the CeA) in close contact to NK3R-immunoreactive cell bodies in a WT(OVX+E2) (left panel) and Kiss1 KO+E2 (right panel). Scale bar: 20 µm. opt: optic tract, MeA: medial amygdala, CeA: central amygdala.

References

1 Clarkson, J. & Herbison, A. E. Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons. Endocrinology 147, 5817-5825, doi:en.2006-0787 [pii]10.1210/en.2006-0787 (2006).
Figure 2

(A) pAAV5-hSvn-DIO-hM3D(Ga)-mCherrv

Kiss1-Cre mouse

ARC

(B) GFP

mCherry

Merge

3V

Scale bar: 100 μm

(C) LH (ng/mL)

Saline

CNO

Minutes after injection

0 15 30 45 60 75 90

LH (ng/mL)

Saline CNO

AUC

(D) Kiss1cre/cre (KO)

LH (ng/mL)

Saline CNO

AUC
Figure 3

A

- NK3R/GnRH
- WTox
- Kiss1KO
- ARC
- 3V

B

- NK3R
- 3V
- ME

C

- NK3R/GnRH
- 3V
- ME
- MS
- WTox+E2
- Kiss1KO(+E2)

D

- WT_{O VX+E2}
- Kiss1KO_{+E2}
- LH (ng/mL)
- AUC

E

- WT_{O VX+E2}
- Kiss1KO_{+E2}
- LH (ng/mL)
- AUC

Data points and error bars are shown for each condition, with significant differences indicated by asterisks (*) and ****.
Figure 4

A

WTovx

WTovx+E2

Kiss1KO

Kiss1KO+E2

Scale bar: 50 μm

C

Number of NK3R-ir cells

|          | WTovx | WTovx+E2 | Kiss1KO | Kiss1KO+E2 |
|----------|-------|----------|---------|------------|
| WT       | 2.5   | 6.0      | 2.0     | 3.0        |
| Kiss1KO  | 1.0   | 2.0      | 1.5     | 2.5        |

D

LH (ng/mL)

|          | WTovx+E2 | Kiss1KO+E2 | Kiss1KO |
|----------|----------|-------------|---------|
| 0        | 0.2      | 0.3         | 0.5     |
| 15       | 1.0      | 1.5         | 1.2     |
| 30       | 2.0      | 2.5         | 2.0     |
| 45       | 3.0      | 3.5         | 3.0     |

AUC

|          | WTovx+E2 | Kiss1KO+E2 | Kiss1KO |
|----------|----------|-------------|---------|
| 0        | 5.0      | 10.0        | 15.0    |
| 15       | 10.0     | 20.0        | 30.0    |
| 30       | 25.0     | 50.0        | 75.0    |

E

pAAV5-hSyn-DIO-hM3D(Gq)-mCherry

Kiss1-Cre mouse

MeA

F

GFP

mCherry

Merge

Scale bar: 50 μm

G

LH (ng/mL)

|          | Saline | CNO |
|----------|--------|-----|
| WTovx+E2 |        |     |
| Kiss1KO+E2 |      |     |
| Kiss1KO  |        |     |

AUC

|          | Saline | CNO |
|----------|--------|-----|
| WTovx+E2 |        |     |
| Kiss1KO+E2 |      |     |
| Kiss1KO  |        |     |

H

LH (ng/mL)

|          | Saline | CNO |
|----------|--------|-----|
| WTovx+E2 |        |     |
| Kiss1KO+E2 |      |     |
| Kiss1KO  |        |     |

AUC

|          | Saline | CNO |
|----------|--------|-----|
| WTovx+E2 |        |     |
| Kiss1KO+E2 |      |     |
| Kiss1KO  |        |     |
