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 (E_2) 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 E_2 levels. Here, we demonstrate that in the presence of E_2, NKB signaling induces LH release in a kisspeptin-independent manner. Moreover, senktide (NKB receptor agonist) delivery to the medial amygdala (MeA) increases LH in E_2-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 ($Kiss1^{AVPV/PeN}$) 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

75 treated with a *Kiss1r* antagonist (Grachev et al., 2012) or agonadal juvenile monkeys with a
76 desensitized *Kiss1r* (Ramaswamy et al., 2011). These studies clearly indicated the importance of
77 NKB signaling onto Kiss1^{ARC} neurons for GnRH/LH secretion.

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

90 The experimental studies described here aimed to assess whether senktide can stimulate
91 LH release, in the presence of E_2, in adult mice that lack a functional kisspeptin signaling system,
92 and if so, investigate the potential mechanisms involved. We used mice in which Cre recombinase
93 was targeted to the *Kiss1* locus and prevented Kiss1 protein synthesis; consequently,
94 homozygous mice (*Kiss1*$^{cre/cre}$) are *Kiss1* KO and display severe hypogonadotropic hypogonadism
95 (Padilla et al., 2018). Our findings reveal a novel kisspeptin-independent pathway of GnRH/LH
96 release in the female mouse, which is activated in the presence of E_2 and involves NKB/NK3R
97 signaling within the medial amygdala (MeA).

98

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 (WT_{ORX}) or ovariectomy (WT_{OVX})] adult WT male and female mice. In the absence of sex steroids [testosterone (T) or E_2, 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 [(WT_{OVX+E2}, WT_{ORX+T}); P=0.007 and P=0.0049, respectively; **Figure 1C, D**]. Interestingly, the same was observed in *Kiss1* KO female mice supplemented with E_2 [(*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 E_2-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 *Kiss1*$^{Cre/+}$ or *Kiss1* KO (i.e. *Kiss1*$^{Cre/Cre}$) 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*$^{Cre/+}$ and *Kiss1 KO* 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$^{Cre/+}$ 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 E_2-supplemented *Kiss1* KO (i.e. *Kiss1*$^{cre/cre}$) 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^{ARC} 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_{OVX} 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 E_2 **(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 E_2, (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_{OVX+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_{OVX+E2} and *Kiss1* KO females supplemented with E_2 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 E_2, *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$^{Cre/+}$ mice expressing hM3Dq:mCherry in the MeA and treated with CNO to activate the Kiss1^{MeA} 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_2-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^{MeA} 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^{MeA} 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

198 and GnRH cell bodies irrespective of the presence or absence of sex steroids. This reveals
199 certain anatomical differences to what has been previously demonstrated in the rat, where
200 ~16% of GnRH cell bodies were found to contain NK3R protein (Krajewski et al., 2005).
201 However, our results agree with reports of no NK3R expression in GnRH neurons of the ewe
202 (Amstalden et al., 2010) suggesting the existence of species differences. Overall, our data
203 suggest that the kisspeptin-independent action of NKB cannot be attributed to direct stimulation
204 of NK3R located on GnRH neurons.

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

218 The distribution of NK3R has been described in the human, rat, and ewe (Mileusnic et al.,
219 1999, Krajewski et al., 2005, Amstalden et al., 2010) and here, we confirm a similar distribution in
220 the mouse brain. Interestingly, in certain areas the immunoreactivity of NK3R-containing cell
221 bodies was highly dependent on sex steroid levels. Specifically, estrogen downregulated NK3R
222 expression in the ARC whereas the opposite was true for the MeA, with more NK3R containing

cell bodies evident when animals were supplemented with E_2. High sensitivity of NK3R expression to E_2, 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 E_2 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 E_2-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 E_2 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 (Comninos 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 $Kiss1^{AVPV/PeN}$ 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 E_2. Upon further investigation, we identified two mechanisms that can lead to GnRH/LH secretion in the female, involving $Kiss1^{MeA}$ 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 $Kiss1^{Cre:GFP}$ 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, $Kiss1^{Cre:Cre}$ 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 *ad libitum* access to tap water. For all studies, C57Bl/6 WT or *Kiss1*$^{Cre/+}$ (heterozygous state) males or females between age 8 and 20 weeks were used and studied in parallel to *Kiss1*$^{Cre/Cre}$ (*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 *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) *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 Kiss1^{ARC} (KNDy) neuron can stimulate LH release, we used a chemogenetic approach to specifically activate Kiss1^{ARC} neurons of *Kiss1*$^{Cre/+}$ or *Kiss1*$^{Cre/Cre}$ mice treated with 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 $3x10^{12}$ 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_{OVX}, WT_{OVX+E_2}, *Kiss1 KO* and *Kiss1* KO_{+E_2} (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_{OVX+E2} and *Kiss1* KO_{+E2} females. Unilateral injections were performed as described below on WT_{OVX+E_2}, and *Kiss1* KO_{+E_2} (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_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

(0) 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 Kiss1^{MeA} neuron can stimulate LH release, we used a similar approach as previously described to specifically activate Kiss1^{MeA} neurons of *Kiss1*$^{Cre/+}$ or *Kiss1*$^{Cre/Cre}$ mice treated with E_2 (n=5/group). E_2 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 *Kiss1*$^{Cre/Cre}$ mice injected with the Cre-dependnet hM3Dq:mCherry in the MeA were also treated with an E_2 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 (WT_{ORX} and WT_{OVX}) with circulating sex steroid levels being restored between genotypes (WT_{ORX+T}, WT_{OVX+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 17*β*-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 preciously 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

424 medial septal nucleus to the caudal part of the retrochiasmatic area (+1.0 mm to -1.0 mm relative
425 to bregma; containing GnRH cell bodies and the RVP3V population of *Kiss1* cells) and the second
426 encompassing the ARC nucleus (from -1.0 till -2.8 mm relative to bregma; containing ARC and
427 amygdala *Kiss1* populations of cells). Sections were stored at -20^{0}C in cryoprotectant [30%
428 sucrose in 0.1 m PB containing 30% ethylene glycol (Thermo Fisher Scientific) and 0.01% sodium
429 azide] until further processing.

430 *General procedures*

431 For all staining procedures detailed below, free-floating sections at room temperature and
432 under gentle agitation were thoroughly washed in PBS, pH 7.4, between all incubations, and
433 treated with 10% H_2O_2 (10 min; in PBS) and PBS$^+$ [1 h; PBS containing 0.1% bovine serum
434 albumin (Thermo Fisher Scientific) and 0.4% Triton X-100 (Sigma-Aldrich)]. Brain sections were
435 mounted onto Superfrost plus glass slides (Fisher Laboratories), air dried and cover-slipped with
436 Vectashield HardSet Mounting Medium (Vector Laboratories). For immunodetection of the
437 different proteins, sections of all animals were processed simultaneously. Secondary antibodies
438 were tested for non-specific binding by primary antibody omission. mCherry immunostaining was
439 compared and validated with the endogenous mCherry expressed by the viral construct in a
440 separate series of sections. The anti-GnRH and anti-NKB antibodies produced staining patterns
441 identical to those from several independent GnRH and NKB antibodies (Merlo et al., 2007,
442 Krajewski et al., 2005) and consistent with those by in situ hybridization (Duarte et al., 2006). The
443 specificity of the NK3R antibody was tested by staining brain tissue collected from NK3R KO mice
444 (True et al., 2015) alongside experimental tissue. No NK3R staining was observed in NK3RKO
445 animals (**Supplemental Figure 2**).

446 *mCherry and GFP.* Brains from *Kiss1*$^{Cre/+}$ and *Kiss1*$^{Cre/Cre}$ mice (n=5/group) injected with
447 AAV5-hSyn-DIO-hM3D(Gq)-mCherry were assessed for mCherry reporter expression to confirm
448 bilateral infection of ARC or MeA GFP expressing *Kiss1* neurons with the AVV carrying Cre-

449 dependent hM3D receptor. One series of free-floating sections containing ARC and amygdala
450 from each animal was incubated overnight in blocking solution containing rat anti-mCherry
451 primary antiserum, Alexa Fluor 594 conjugate (1:500; Thermo Fisher, Cat. No. M11240). The next
452 morning sections were extensively washed in PBS and then incubated overnight in rabbit anti-
453 GFP tag antibody (1:5,000; Thermo Fisher, Cat. No. A-6455) followed by goat-anti-rabbit DyLight
454 488 secondary antibody (1:200; Thermo Scientific, Cat. No. 35552).

455 *NK3R and GnRH.* Brains from WT and Kiss1 KO mice were processed for NK3R and
456 GnRH in order to compare the protein distribution of NK3R in the two genotypes and investigate
457 the potential colocalization with GnRH in the hypothalamus. Furthermore, we processed tissue
458 sections from $WT_{(OVX)}$, $WT_{(OVX+E2)}$, *Kiss1 KO* and *Kiss1 KO*$_{+E2}$ groups to determine whether E_2
459 regulates protein expression of NK3R in the a) preoptic area/medial septum, where most of the
460 GnRH cell bodies are located b) ARC where KNDy cells reside, and c) MeA where senktide
461 induced LH release. Hence, a series of every third section, extending from the level of the optic
462 chiasma to the mammillary nucleus, was processed for NK3R and GnRH using a modified
463 protocol previously described (Goodman et al., 2007). Tissue sections were incubated
464 sequentially with: 1) rabbit anti-NK3R (1:30,000; Novus Biologicals, Cat. No. NB300-102) for 17
465 hours, 2) biotinylated goat anti rabbit IgG (1:500; Vector Laboratories, Cat. No. BA1000), for 1
466 hour, 3) avidin and biotinylated horseradish peroxidase complex (Avidin-Biotin Complex; 1:500;
467 Vector Laboratories, Cat. No. PK-6100) for 1 hour, 4) biotinylated tyramine (1:250; PerkinElmer,
468 Cat. No. NEL700A001KT), containing 0.003% H_2O_2 for 10 minutes, and 5) DyLight 488
469 conjugated streptavidin (1:200; Thermo Fisher, Cat. No. 35552) for 30 minutes. Next, sections
470 were incubated with rabbit anti-GnRH (1:1,000; Abcam; Cat. No. ab5617) for 17 hours. The next
471 morning, sections were washed and incubated with goat anti-rabbit Alexa 555 (1:200; Thermo
472 Fisher, Cat. No. A-21428) for 30 minutes. The specificity of the NK3R antibody we used in our
473 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

498 magnification containing ARC and MeA, to determine the effect of E_2 on protein expression in
499 these areas. Counts were averaged per mouse, per brain area. A digital camera (CoolSnap EZ,
500 PhotometricsTM, Canada) attached to a microscope (Nikon Eclipse 90i), with the appropriate
501 excitation for DyLight 488 (green flurescence) and Alexa 555 (red fluorescence) and NIS-
502 Elements Viewer AR 310 software was used to examine tissue sections and superimpose two
503 images and determine putative colocalization or interactions. Montages of images and
504 adjustments of brightness and contrast levels were made in Adobe Photoshop CS5.

505 **Statistics**

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

512 **Study Approval**

513 All animal care and experimental procedures were approved by the National Institute of
514 Health, and Brigham and Women's Hospital Institutional Animal Care and Use Committee,
515 protocol #05165. The Brigham and Women's Hospital is a registered research facility with the
516 U.S. Department of Agriculture (#14-19), is accredited by the American Association for the
517 Accreditation of Laboratory Animal Care and meets the National Institutes of Health standards as
518 set forth in the Guide for the Care and Use of Laboratory Animals (DHHS Publication No. (NIH)
519 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 *Kiss1*^{Cre} mice and validated the *Kiss1 KO* 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 *Kiss1* KO 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 (WT_{ORX} and WT_{OVX}, respectively) and studied in parallel to hypogonadal *Kiss1 KO* littermates (n=10/group). **(C. D)** LH levels in WT and *Kiss1 KO* male and female mice with restored levels of sex steroids (WT_{ORX+T}, WT_{OVX+E2}, *Kiss1* KO_{+T}, *Kiss1* KO_{+E2}; n=10/group). Paired *t*-test ****P<0.0001, **P<0.007 compared to basal levels of LH. T=testosterone, E_2=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 *Kiss1 KO* 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

545 *Kiss1*$^{Cre/+}$ (**C**) and *Kiss1*$^{Cre/Cre}$ (KO **D**) female mice (n=5-8/group). 3V: third ventricle. **P*<0.035,
546 ***P*<0.006, ****P*<0.0001.
547
548 **Figure 3.** Representative photomicrographs depicting dual label detection of NK3R (green) and
549 GnRH (red) in the ARC (**A**) and ME (**B**) of WTovx and *Kiss1 KO* animals. (**A**) Panels on the right
550 are enlarged images (scale bar: 20 μm) from boxed areas on the left (scale bar: 100 μm) showing
551 numerous close appositions (arrowheads) between NK3R and GnRH in the ARC. (**B**) Enlarged
552 images of the boxed area from the *Kiss1 KO* animal in (**A**) showing intense GnRH but lack of
553 NK3R staining in the ME. (**C**) Dual-label detection of NK3R (green) and GnRH (red) within the
554 region of the MS of E_2-supplemented WT and *Kiss1 KO* animals [WT_{OVX+E2} and *Kiss1 KO*$_{+E2}$,
555 respectively; scale bar: 50 μm]. Arrowheads indicate sites of close apposition. (**D, E**) Mean ± SEM
556 LH responses and area under the curve (AUC) to an injection of senktide into the ARC (**D**) or
557 POA (**E**) of WT_{OVX+E2} (blue line-blue bar) and *Kiss1 KO*$_{+E2}$ (green line-green bar) female mice
558 (n=5/group). 3V: third ventricle, ARC: arcuate, ME: median eminence, MS: medial septum.
559 ***P*<0.0015, *****P*<0.0001.
560
561 **Figure 4. (A)** Representative photomicrographs depicting NK3R-immunoreactive cell bodies and
562 fibers in the MeA of (**A**) WT_{OVX} (left panel) and WT_{OVX+E2} (right panel) or *Kiss1 KO* (left panel) and
563 *Kiss1 KO*$_{+E2}$ (right panel). (**C**) Mean ± SEM number of NK3R-immunoreactive cells per 30 *μm*
564 section in the MeA of WT_{OVX}, WT_{OVX+E2}, *Kiss1 KO* and *Kiss1 KO*$_{+E2}$ animals (6-8 sections from 5
565 animals/group). (**E**) Schematic representation of the site of injection of an AAV encoding a Cre-
566 dependent hM3Dq:mCherry. **(B)** Representative photomicrograph of a coronal brain section
567 stained for GFP (green), mCherry (red) and merged GFP and mCherry immunoreactivity in the
568 MeA of a *Kiss1 KO* female mouse >3 weeks after hM3Dq:mCherry injection (Scale bar 50 μm).
569 (**G, H**) Mean ± SEM LH responses and area under the curve (AUC) to an injection of saline (grey

570 line-empty bar) or CNO (green line-green bar) of hM3Dq DREADD injected *Kiss1*$^{Cre/+}$ (**G**) and
571 *Kiss1*$^{Cre/Cre}$ (KO; **H**) female mice (n=5/group). opt: optic tract. **P*<0.025, ***P*<0.0014.
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719

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 *Kiss1*$^{Cre/+}$ and *Kiss1*$^{Cre/Cre}$ 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

Kiss1 neurons in *Kiss1 KO* mice. The anatomical relationship between the two proteins appeared to be similar in both genotypes (*Kiss1*$^{Cre/+}$ 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*$^{Cre/+}$ (**A**) and *Kiss1*$^{Cre/Cre}$ (**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 E_2 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

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Figure 1

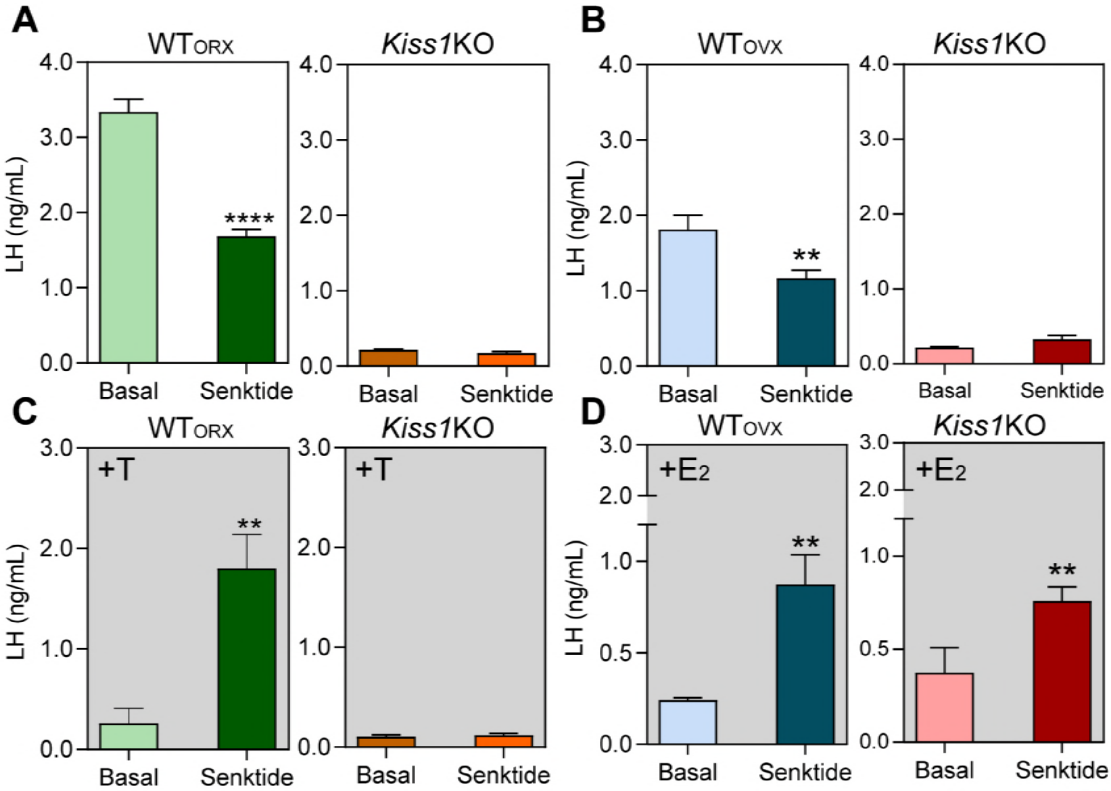

A
WTORX
Kiss1KO
LH (ng/mL)
4.0
3.0
2.0
1.0
0.0
****
Basal
Senktide
B
WTOVX
Kiss1KO
**
C
+T
D
+E2

Figure 2

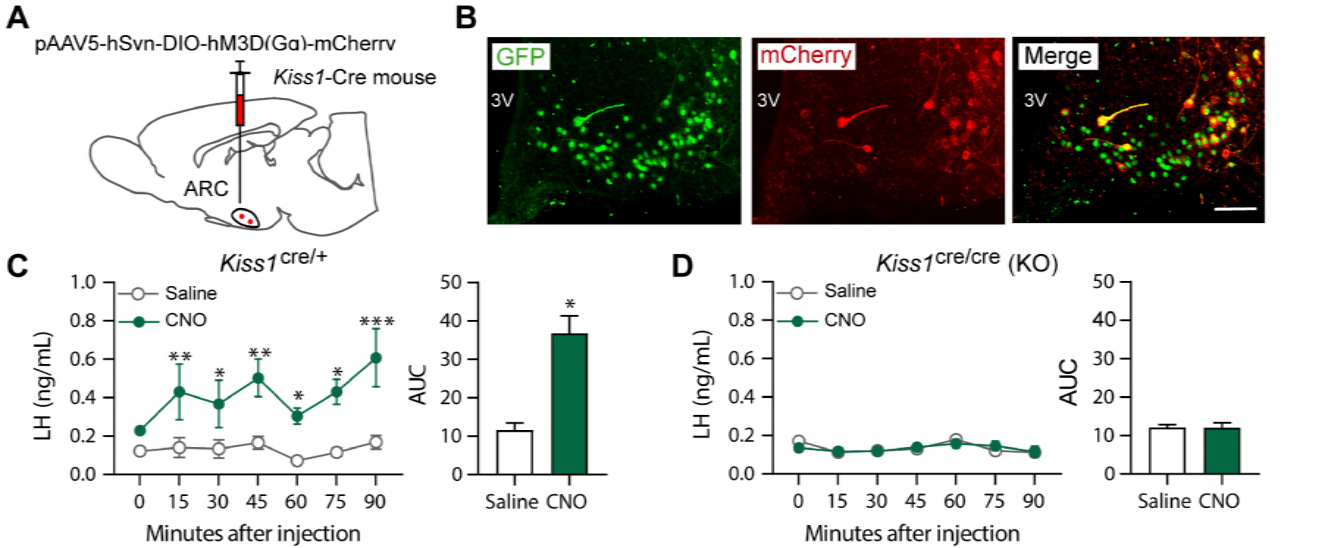

A
pAAV5-hSyn-DIO-hM3D(Gq)-mCherry
Kiss1-Cre mouse
ARC
B
GFP
3V
mCherry
3V
Merge
3V
C
Kiss1cre/+
Saline
CNO
LH (ng/mL)
Minutes after injection
AUC
Saline
CNO
D
Kiss1cre/cre (KO)
Saline
CNO
LH (ng/mL)
Minutes after injection
AUC
Saline
CNO

Figure 3

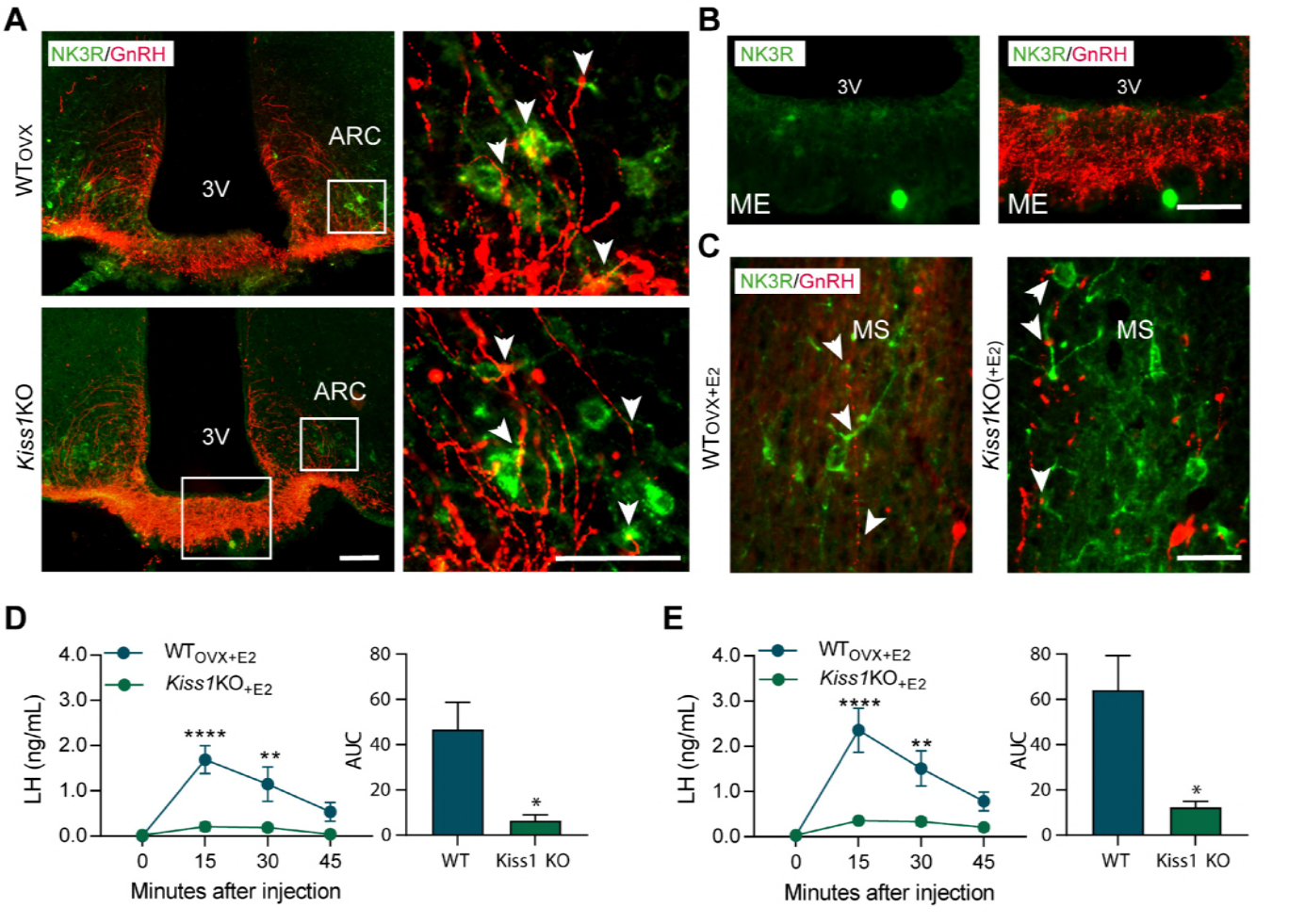
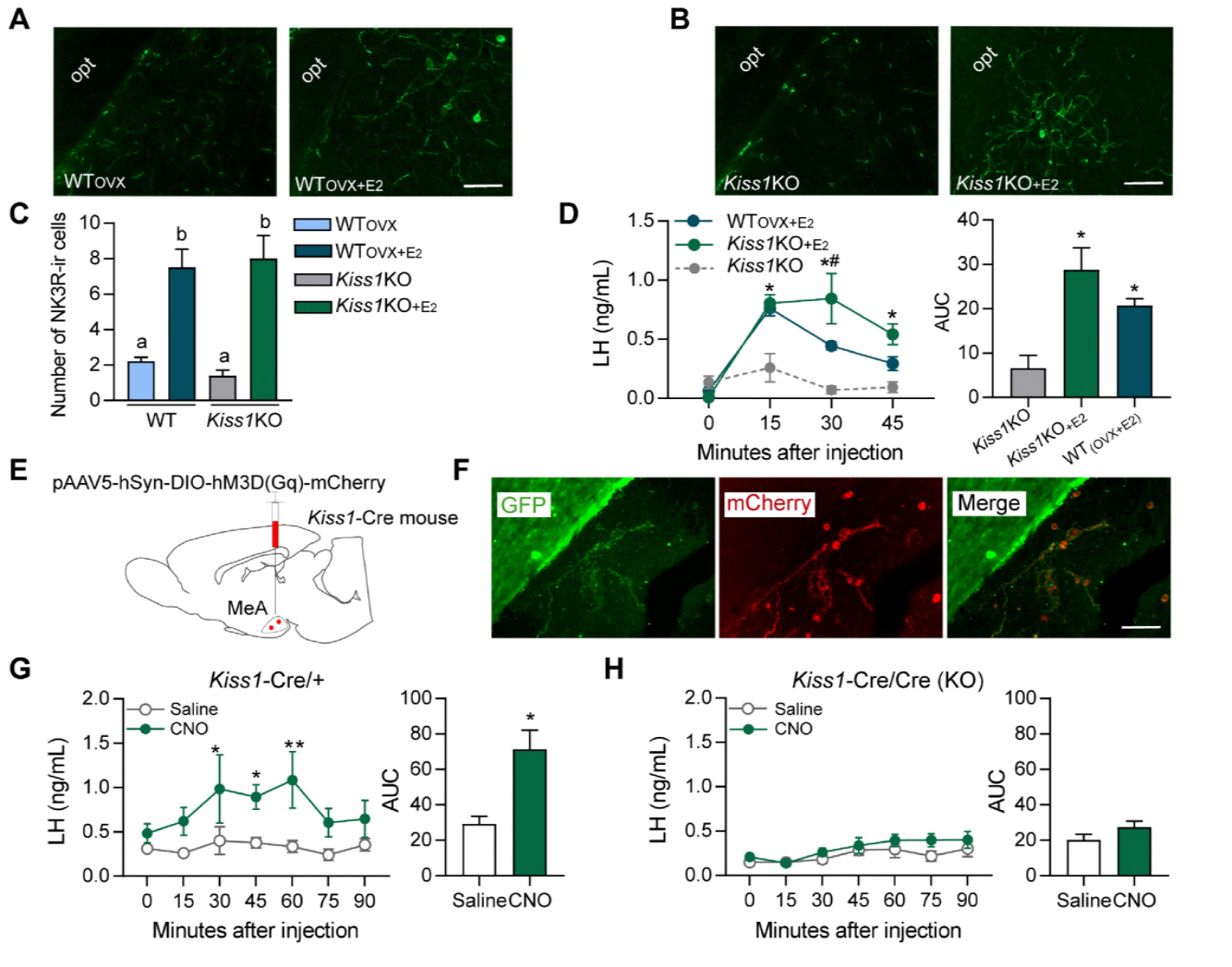
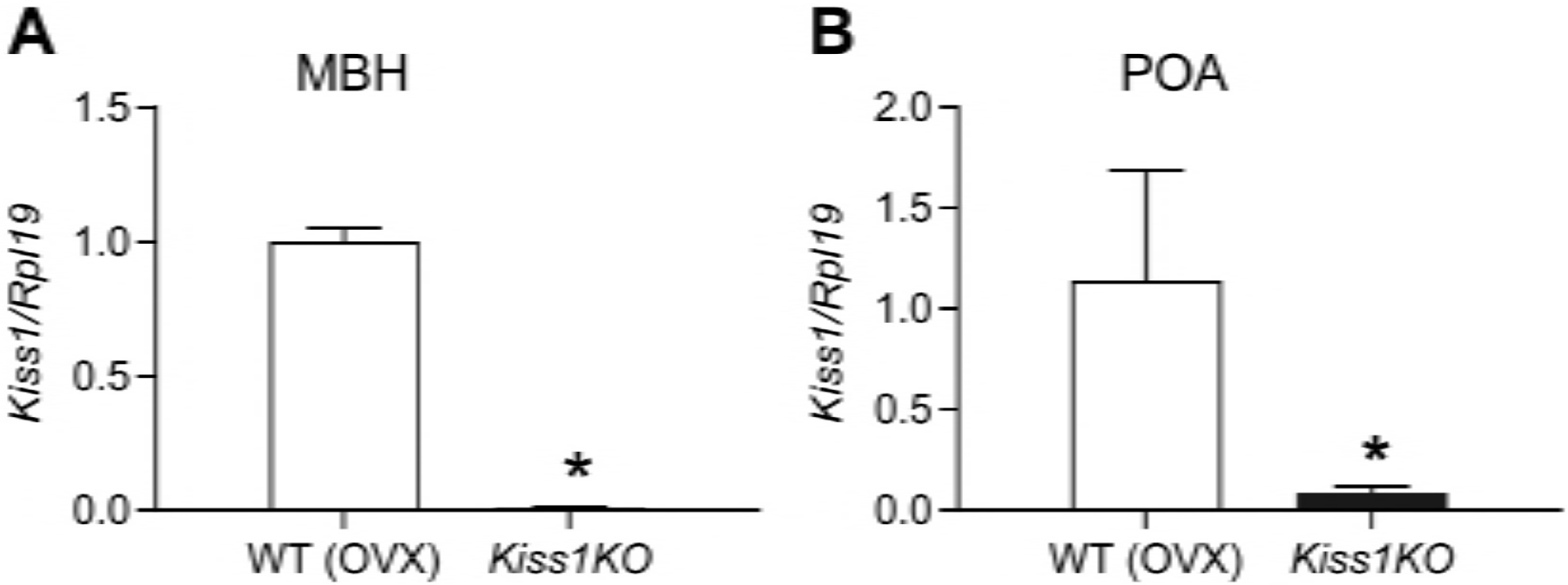

A
NK3R/GnRH
WTOVX
ARC
3V
Kiss1KO
B
NK3R
3V
ME
NK3R/GnRH
C
MS
WTOVX+E2
Kiss1KO(+E2)
D
WTOVX+E2
Kiss1KO+E2
LH (ng/mL)
Minutes after injection
AUC
WT
Kiss1 KO
E

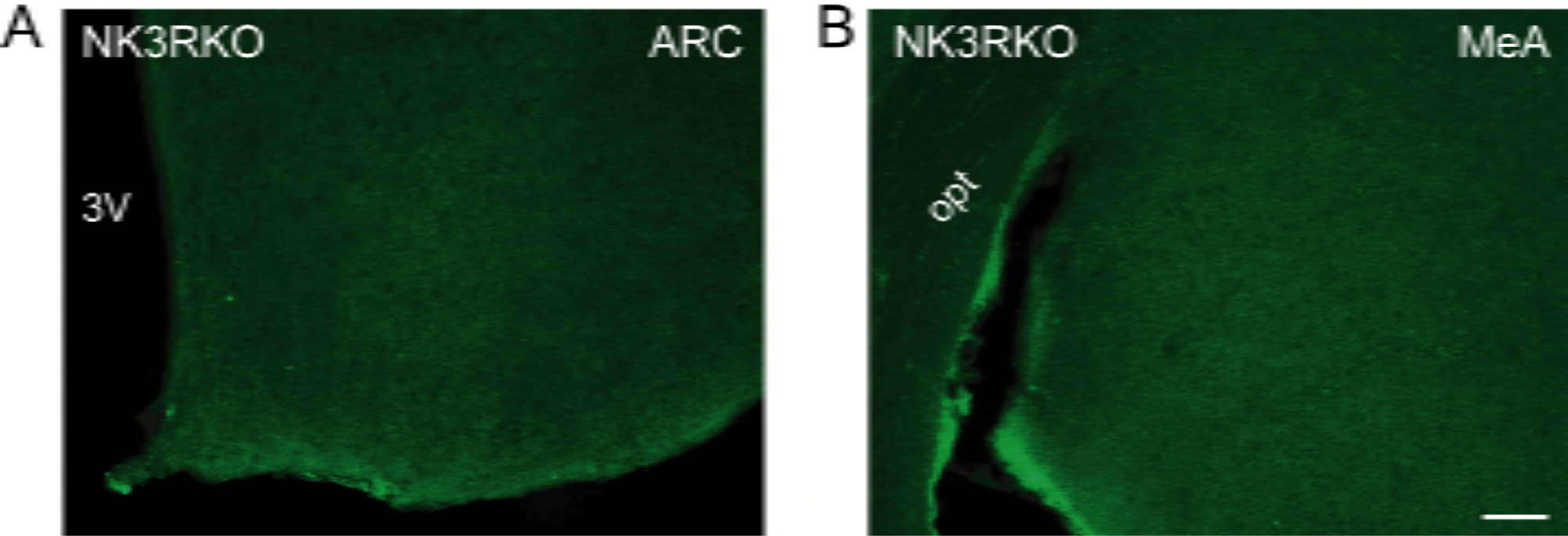
Figure 4


A
opt
WTOVX
opt
WTOVX+E2
B
opt
Kiss1KO
opt
Kiss1KO+E2
C
Number of NK3R-ir cells
WTOVX
WTOVX+E2
Kiss1KO
Kiss1KO+E2
WT
Kiss1KO
D
LH (ng/mL)
Minutes after injection
AUC
Kiss1KO
Kiss1KO+E2
WT (OVX+E2)
E
pAAV5-hSyn-DIO-hM3D(Gq)-mCherry
Kiss1-Cre mouse
MeA
F
GFP
mCherry
Merge
G
Kiss1-Cre/+
Saline
CNO
LH (ng/mL)
Minutes after injection
AUC
Saline
CNO
H
Kiss1-Cre/Cre (KO)
Saline
CNO
LH (ng/mL)
Minutes after injection
AUC
Saline
CNO

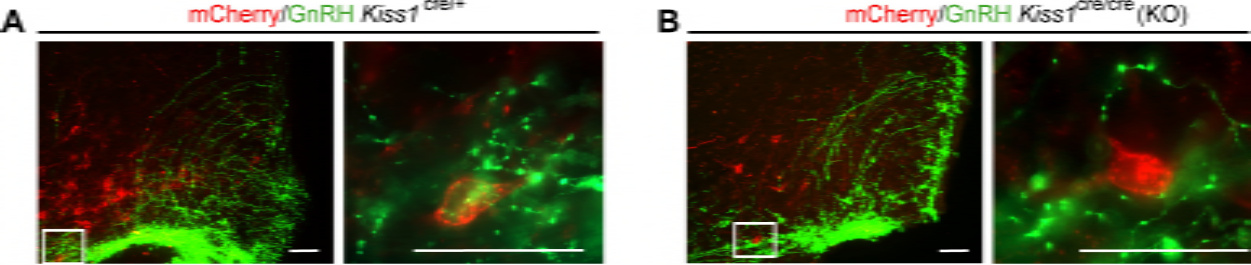
Supplemental Figure 1



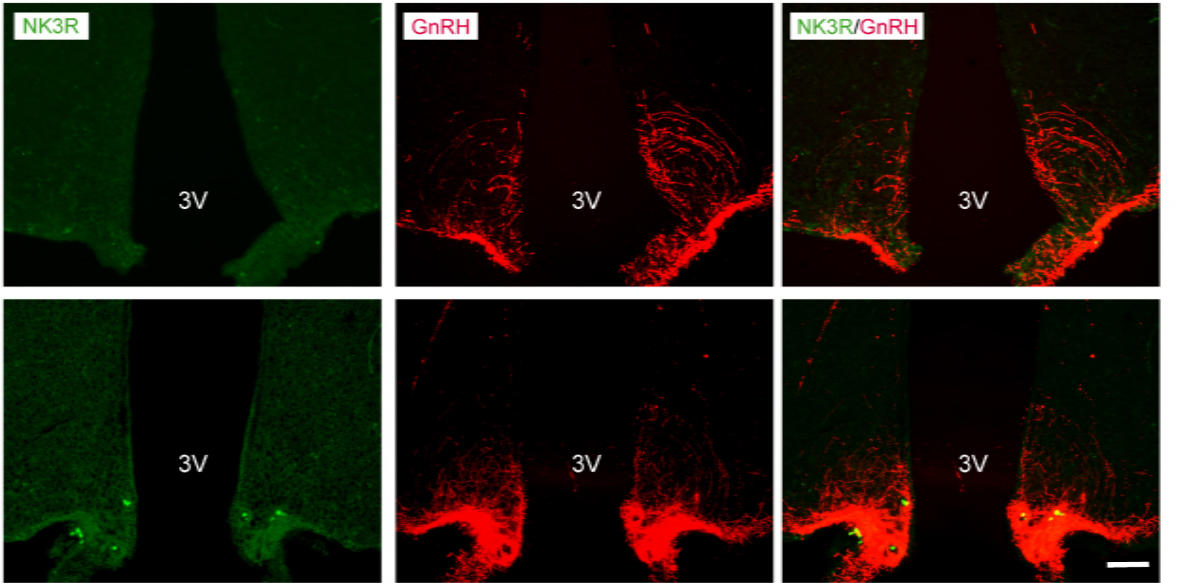
Supplemental Figure 2



Supplemental Figure 3



Supplemental Figure 4



Supplemental Figure 5