

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

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## 27 **Abstract**

28 Neurokinin B (NKB) signaling is critical for reproduction in all studied species. The existing  
29 consensus is that NKB induces GnRH release via kisspeptin (*Kiss1*) stimulation in the arcuate  
30 nucleus. However, the stimulatory action of NKB is dependent on circulating estrogen (E<sub>2</sub>) levels,  
31 without which, NKB inhibits LH release. Importantly, the evidence supporting the kisspeptin-  
32 dependent role of NKB, derives from models of persistent hypogonadal state [e.g. *Kiss1r* knock-  
33 out (KO) mice], with reduced E<sub>2</sub> levels. Here, we demonstrate that in the presence of E<sub>2</sub>, NKB  
34 signaling induces LH release in a kisspeptin-independent manner. Moreover, senktide (NKB  
35 receptor agonist) delivery to the medial amygdala (MeA) increases LH in E<sub>2</sub>-treated *Kiss1* KO  
36 females (but not males or sham-treated females) similar to controls, and thus, this increase is  
37 independent of *Kiss1* neurons. These results document a novel kisspeptin-independent  
38 regulatory pathway of reproductive function in females mediated by NKB-responsive neurons in  
39 the MeA.

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## 50 Introduction

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

57           Kisspeptins, secreted from *Kiss1* neurons in the arcuate (*Kiss1*<sup>ARC</sup>) and anteroventral  
58 periventricular/periventricular (*Kiss1*<sup>AVPV/PeN</sup>) nuclei, have been directly linked to the release of  
59 gonadotropin releasing hormone [GnRH (Smith et al., 2006, Fergani and Navarro, 2016)]  
60 however, there is another population of *Kiss1* neurons in the medial amygdala (*Kiss1*<sup>MeA</sup>) whose  
61 function has not yet been elucidated (Fergani and Navarro, 2016, Smith et al., 2006, Pineda et  
62 al., 2017). In addition to kisspeptin, *Kiss1*<sup>ARC</sup> neurons express neurokinin B and dynorphin and  
63 these neurons are sometimes referred to as KNDy neurons (Fergani and Navarro, 2016).  
64 Functional studies in mice and other species suggest that the stimulatory effect of NKB/NK3R  
65 signaling lies up-stream of *Kiss1*/*Kiss1r*, which in turn, directly activates GnRH neurons and,  
66 hence, stimulates luteinizing hormone (LH) and follicle stimulating hormone (FSH) secretion into  
67 the peripheral circulation (Fergani and Navarro, 2016). Specifically, NKB signaling onto kisspeptin  
68 occurs via the auto-synaptic activation of NK3R residing on *Kiss1*<sup>ARC</sup> neurons (Fergani and  
69 Navarro, 2016). The existence of this pathway is supported by the fact that the selective NK3R  
70 agonist, senktide, induces Fos expression in *Kiss1*<sup>ARC</sup> neurons *in vivo* (Navarro et al., 2011a) and  
71 increases of their electrical activity in hypothalamic slices (de Croft et al., 2013, Navarro et al.,  
72 2011b). Subsequently, kisspeptin signaling was deemed an indispensable part of the reproductive  
73 role of NKB, as the stimulatory effect of senktide on LH secretion was shown to be absent in  
74 *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 gonadal juvenile monkeys with a  
76 desensitized *Kiss1r* (Ramaswamy et al., 2011). These studies clearly indicated the importance of  
77 NKB signaling onto *Kiss1*<sup>ARC</sup> 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<sub>2</sub>, 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*<sup>cre/cre</sup>) 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<sub>2</sub> and involves NKB/NK3R  
97 signaling within the medial amygdala (MeA).

98

## 99 **Results**

### 100 ***Central (ICV) administration of senktide stimulates LH release in female Kiss1 KO mice in*** 101 ***the presence of sex steroids.***

102 To investigate potential kisspeptin-independent stimulation of LH after central activation  
103 of NK3R signaling with senktide, we compared hypogonadal *Kiss1* KO mice (Padilla et al., 2018)  
104 of both sexes to gonadectomized [GNX; orchidectomy (WT<sub>ORX</sub>) or ovariectomy (WT<sub>OVX</sub>)] adult WT  
105 male and female mice. In the absence of sex steroids [testosterone (T) or E<sub>2</sub>, in males and  
106 females, respectively] ICV senktide administration decreased plasma LH levels by ~50% in WT  
107 males ( $P < 0.0001$ ) and ~36% in WT females ( $P = 0.0016$ ) with no alteration observed in *Kiss1* KO  
108 mice of either sex (**Figure 1A, B**). When circulating levels of sex steroids were restored, LH  
109 release was significantly increased in WT males and females [(WT<sub>OVX+E2</sub>, WT<sub>ORX+T</sub>);  $P = 0.007$  and  
110  $P = 0.0049$ , respectively; **Figure 1C, D**]. Interestingly, the same was observed in *Kiss1* KO female  
111 mice supplemented with E<sub>2</sub> [(*Kiss1* KO<sub>+E2</sub>);  $P = 0.005$ ; **Figure 1D**] but not in male *Kiss1* KO mice  
112 supplemented with T (*Kiss1* KO<sub>+T</sub>; **Figure 1C**) revealing the existence of a female-specific,  
113 kisspeptin-independent but E<sub>2</sub>-dependent, NKB/NK3R signaling pathway that controls LH  
114 release.

### 115 ***Chemogenetic activation of the ARC KNDy neuron stimulates LH release in control but not*** 116 ***Kiss1 KO female mice.***

117 A Cre-dependent activating DREADD (hM3D-Gq) tagged with mCherry and packaged in  
118 an adeno-associated virus (serotype 5; **Figure 2A**) was injected into the ARC of *Kiss1*<sup>Cre/+</sup> or *Kiss1*  
119 KO (i.e. *Kiss1*<sup>Cre/Cre</sup>) mice. Analysis following the completion of pharmacological studies  
120 demonstrated that mCherry expression was present throughout the ARC (**Figure 2B**) and not  
121 elsewhere. There was limited variability in the spread of mCherry among animals which extended  
122 throughout the medial-caudal extent of the ARC (approximately -1.40 mm to -2.30 mm from  
123 bregma; paxinos atlas). Six out of twenty mice (distributed among the groups) had primarily

124 unilateral m-Cherry spread, but they did not differ significantly from their respective groups in LH  
125 concentrations, and were therefore, included in further analyses. Within the ARC, of both  
126 *Kiss1<sup>Cre/+</sup>* and *Kiss1 KO* genotypes, HM3D:mCherry was expressed in ~32 % of GFP-  
127 immunoreactive cells (expressed in Kiss1 neurons in this mouse model), and was not observed  
128 in non-GFP cells or other brain areas.

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

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

139 The distribution of NK3R was investigated in WT and *Kiss1 KO* mouse brain sections  
140 (**Figure 3, 4 and Supplemental Figure 4 and 5**). Large NK3R immunoreactive neurons and  
141 fibers were identified in the substantia innominata, paraventricular nucleus, supraoptic nucleus,  
142 lateral hypothalamus, zona incerta, and perifornical regions. Interestingly, in the ARC, cells  
143 containing NK3R were evident only in WT<sub>OVX</sub> and hypogonadal *Kiss1 KO* female mice (**Figure 3A**  
144 **and Supplemental Figure 4**), whereas, in the MeA, NK3R cell bodies appeared only when WT  
145 and *Kiss1 KO* females were supplemented with E<sub>2</sub> (**Figure 4A, 4B and 4C**).

146 Next, we investigated the anatomical relationship of NK3R and GnRH expression with  
147 dual-label immunohistochemistry, which revealed close appositions between the two proteins in  
148 the ARC and medial septum (MS) (**Figure 3A, 3C**). In these two areas, GnRH cells and fibers

149 showed dense intermingling and multiple foci of close apposition with NK3R containing cells and  
150 fibers, but no co-expression within GnRH cell bodies (>100 cells analyzed from a total of 16 mice;  
151 **Figure 3C**). Interestingly, we observed no fibers containing NK3R immunoreactivity in the internal  
152 or external zone of the median eminence (ME; **Figure 3A, 3B**).

153 To identify the brain area in which NK3R receptive neurons that mediate the kisspeptin-  
154 independent GnRH release reside, we stereotaxically administered senktide specifically into the  
155 ARC, the POA (at the level of the MS) or the MeA. These areas are prime candidates to play a  
156 role in LH stimulation because (a) they contain NK3R, the immunoreactivity of which is regulated  
157 by E<sub>2</sub>, (b) there is an anatomical overlap of NK3R and GnRH protein, at least in the MS and ARC  
158 and (c) they contain GnRH and/or Kiss1 cell bodies and fibers and are known to play an important  
159 role in reproductive function (Smith et al., 2006, Kim et al., 2011). Senktide administration into the  
160 ARC or POA of WT<sub>OVX+E<sub>2</sub></sub> mice stimulated LH secretion within 15 min from drug infusion (**Figure**  
161 **3D, 3E**;  $P < 0.0001$  for both) compared to *Kiss1* KO animals. However, when senktide was  
162 administered into the MeA of WT<sub>OVX+E<sub>2</sub></sub> and *Kiss1* KO females supplemented with E<sub>2</sub> a robust  
163 increase in LH was observed within 15 min after senktide infusion that was similar in both  
164 genotypes (**Figure 4D**). Conversely, in the absence of E<sub>2</sub>, *Kiss1* KO females did not show any  
165 alteration in LH release (**Figure 4D**), mimicking the LH responses we obtained after an ICV  
166 injection of senktide in these animals (**Figure 1B**).

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

169 Similar to the experiments described above, we delivered Cre-dependent AAV5-DIO-  
170 hM3Dq:mCherry to the MeA of *Kiss1*<sup>Cre/+</sup> or *Kiss1*<sup>Cre/Cre</sup> mice. HM3Dq:mCherry expression was  
171 present in the MeA (**Figure 4F**) and was limited to sections ranging from (from -1.6 mm to -2.0  
172 mm from bregma; paxinos atlas). Two out of eight mice (one from each group) had primarily  
173 unilateral spread of the DREADD, but they did not differ significantly from their respective groups

174 in LH concentrations, and were therefore, included in further analyses. Within the MeA, mCherry  
175 cell bodies were co-expressed in ~89 % of GFP-immunoreactive cells (i.e. Kiss1 cells), and was  
176 not observed in non-GFP cells.

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

## 182 Discussion

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

194 From a mechanistic point of view, the most likely kisspeptin-independent pathway for LH  
195 stimulation by NKB would involve the direct regulation of GnRH release (Krajewski et al., 2005).  
196 Despite there being an anatomical overlap of GnRH and NK3R protein, specifically in the ARC  
197 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*<sup>ARC</sup> (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,  $\gamma$ -aminobutyric acid (Skrapits et al., 2015) can  
213 potentially stimulate LH secretion and must also be considered. However, activation of the  
214 *Kiss1*<sup>ARC</sup> (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*<sup>ARC</sup> (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

223 cell bodies evident when animals were supplemented with E<sub>2</sub>. High sensitivity of NK3R expression  
224 to E<sub>2</sub>, has also been reported for the ARC with *in situ* hybridization studies (Navarro et al., 2009).  
225 Interestingly, this regulation of NK3R expression is reminiscent of the regulation of *Kiss1* by E<sub>2</sub> in  
226 these areas (Kim et al., 2011, Smith et al., 2006).

227         Based on the aforementioned anatomical observations we proceeded with senktide  
228 administration into the ARC, POA (at the level of the MS) and MeA of E<sub>2</sub>-treated animals in order  
229 to locate the kisspeptin-independent, LH-stimulating population of NK3R-expressing neurons.  
230 Senktide administration into the ARC or POA (at the level of the MS) significantly stimulated LH  
231 secretion in WT females compared to *Kiss1* KO animals. Therefore, our data suggest that LH  
232 release, as a result of NKB/NK3R signaling in the POA or ARC, is predominantly achieved via  
233 initial kisspeptin release, involving Kiss1<sup>AVPV/PeN</sup> and/or Kiss1<sup>ARC</sup> neuron activation. Indeed, 10%  
234 of Kiss1<sup>AVPV/PeN</sup> and virtually all Kiss1<sup>ARC</sup> neurons contain NK3R (Navarro et al., 2015). Moreover,  
235 both populations are interconnected, Kiss1<sup>ARC</sup> cells project to Kiss1<sup>AVPV/PeN</sup> neurons and GnRH  
236 cell bodies and terminals (Yip et al., 2015), which could also account for the increase in LH after  
237 administration of senktide into the POA. However, a slight increase in LH occurred in the absence  
238 of kisspeptin signaling (*Kiss1* KO mice). In the ARC and MS of the POA, we observed several  
239 instances where NK3R containing fibers were in close apposition to GnRH cell bodies and/or  
240 processes indicating a potential presynaptic action of NKB onto GnRH. It is possible that NKB (or  
241 senktide) signaling onto presynaptic NK3R, results in the enhanced secretion of other stimulatory  
242 neuropeptides, which in turn stimulate GnRH secretion and could account for the slight increase  
243 in LH observed in *Kiss1* KO mice, as recently documented in the rat striatum, in which tachykinins  
244 (including NKB) presynaptically stimulate the release of dopamine (Glowinski et al., 1993).

245         Interestingly, senktide administration into the MeA of females supplemented with E<sub>2</sub>  
246 produced a similar robust increase in LH in animals with or without the presence of the *Kiss1*  
247 gene. Conversely, hypogonadal *Kiss1* KO females did not show any alteration in LH release,

248 indicating that this kisspeptin-independent NKB/NK3R signaling mechanism in the MeA becomes  
249 activated only when E<sub>2</sub> is present. This notion is further supported by our finding that the number  
250 of NK3R cells increases with E<sub>2</sub>, and this upregulation is specific to the MeA. NK3R<sup>MeA</sup> expressing  
251 cells do not co-localize with NKB, but are surrounded by a plethora of NKB fibers (**Supplemental**  
252 **Figure 5**) the source of which remains to be determined. Likely candidates include NKB-  
253 expressing cells residing within the ARC or the neighboring NKB population in the central  
254 amygdala (CeA) (**Supplemental Figure 5**) to date, implicated only in the modulation of fear  
255 memories (Andero et al., 2016).

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

268 The functional relevance of either the MeA kisspeptin-dependent or the kisspeptin-  
269 independent neuronal population that can induce LH release is unknown. A reasonable  
270 hypothesis is that Kiss1<sup>MeA</sup> and NK3R<sup>MeA</sup> neurons are part of the neuronal network linking  
271 pheromonal/social cues and gonadotropin release (Yang et al., 2018). Indeed, estrogen receptors  
272 are expressed in the MeA (Lymer et al., 2018) and the brain region is a central hub for processing

273 sensory inputs such as olfactory signals and integrating these into behavioral (Rajendren and  
274 Moss, 1993, Adekunbi et al., 2018) and neuroendocrine outputs (Pineda et al., 2017). Specifically,  
275 it is compelling to hypothesize that the kisspeptin-independent, NK3R-dependent pathway is  
276 employed for the generation and/or enhancement of the LH surge and/or female sexual behavior,  
277 e.g. lordosis, given that this mechanism was absent in male mice, and is exclusively activated in  
278 the presence of estrogen, similar to what is observed in the female AVPV/PeN (Smith et al., 2006).  
279 In accordance, recent evidence demonstrated the enhancement of the LH surge in rats exposed  
280 to male-soiled bedding, which was accompanied by an increased Fos expression in Kiss1<sup>AVPV/PeN</sup>  
281 neurons as well as various limbic structures, including the MeA (Watanabe et al., 2017, Hellier et  
282 al., 2018).

283 In summary, we have shown that the MeA is a previously unknown component of the  
284 gonadotropic axis. Initially, we observed that senktide administration into the lateral ventricle  
285 stimulates LH release into the peripheral circulation of female mice lacking kisspeptin (*Kiss1* KO),  
286 but only when they are supplemented with E<sub>2</sub>. Upon further investigation, we identified two  
287 mechanisms that can lead to GnRH/LH secretion in the female, involving Kiss1<sup>MeA</sup> or NK3R-  
288 expressing neurons located in the MeA. Collectively, these data demonstrate that the  
289 gonadotropic axis is subject to regulation by signalling originating outside the hypothalamus and  
290 specifically the MeA, involved in the regulation of social behaviors including sexual behavior,  
291 anxiety, and olfaction.

## 292 **Methods**

293

### 294 **Animals**

295 A *Kiss1*<sup>Cre:GFP</sup> knock-in mouse (version 2) was generated from C57Bl/6 blastocysts and  
296 verified at the University of Washington (Padilla et al., 2018). The homozygous version of this  
297 mouse, *Kiss1*<sup>Cre:Cre</sup> is a *Kiss1* KO as characterized elsewhere (Padilla et al., 2018). *Kiss1* gene  
298 was also confirmed undetectable from POA and MBH tissue (**Supplement Figure 1**). Animals

299 were group housed according to sex and bred under constant conditions of temperature (22–  
300 24°C) and light [12 h light (06:00)/dark (18:00) cycle], fed with standard mouse chow (Teklad F6  
301 Rodent Diet 8664) and were given *ad libitum* access to tap water. For all studies, C57Bl/6 WT or  
302 *Kiss1<sup>Cre/+</sup>* (heterozygous state) males or females between age 8 and 20 weeks were used and  
303 studied in parallel to *Kiss1<sup>Cre/Cre</sup>* (*Kiss 1* knock-out state) littermates. In order to test the specificity  
304 of the NK3R antibody, NK3RKO mice were used as described below.

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

307 In this experiment we aimed to assess whether central activation of NK3R signaling with  
308 senktide (an NK3R specific agonist), can stimulate LH release in *Kiss1 KO* mice (i.e., in a  
309 kisspeptin-independent manner) in the presence or absence of sex steroids. Adult WT male and  
310 female mice were GND and studied in parallel to hypogonadal (with low sex steroid levels) *Kiss1*  
311 *KO* littermates (n=10/group). ICV injections (see below) of senktide (Tocris Bioscience, Cat. No.  
312 1068; 600 pmol diluted in 5µl 0.9% NaCl) were performed and blood samples were collected  
313 before (basal) and 25 min after ICV injection for LH measurements as has been previously  
314 described (Navarro et al., 2015). Next, animals were implanted with sex steroids (n=10/group)  
315 and the ICV experiment was repeated a week later. The dose of senktide used, and the time of  
316 blood collection were selected based on our previous studies (Navarro et al., 2015).

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

319 In order to determine whether the release of other components, besides kisspeptin, within  
320 the *Kiss1<sup>ARC</sup>* (KNDy) neuron can stimulate LH release, we used a chemogenetic approach to  
321 specifically activate *Kiss1<sup>ARC</sup>* neurons of *Kiss1<sup>Cre/+</sup>* or *Kiss1<sup>Cre/Cre</sup>* mice treated with E<sub>2</sub> (n=5-  
322 8/group). Females received bilateral stereotaxic injections (see below) of an adeno-associated  
323 virus (pAAV) encoding a Cre-driven Gq-coupled DREADD (pAAV5/hSyn-DIO-hm3Dq:mCherry;

324 Addgene, Cat. No.44361-AAV5; titer  $3 \times 10^{12}$  genome copies per ml; 1  $\mu$ l per hemisphere).  
325 Following infection, mice were given 3 weeks for recovery and maximum expression of the AAV  
326 vector. On the day 1 of the experiment animals were administered an ip bolus injection of vehicle  
327 saline (0.9% NaCl; day 1) and then hM3D receptors were activated by ip injection of its agonist,  
328 clozapine N-oxide (CNO; 10 mg/kg dissolved in saline; day 2). Blood samples were collected just  
329 before saline or CNO treatment (0) and then every 15 min for 90 min. The dose of CNO was  
330 chosen based on previous behavioral studies using hM3Dq manipulations (Ben-Shaanan et al.,  
331 2016). At the end of the experiment, all mice were treated with an icv injection of senktide, as a  
332 control, and to confirm that animals were appropriately treated and primed.

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

335 In this set of experiments, we aimed to locate the brain area which senktide is acting to  
336 stimulate LH release. To this end, we first conducted neuroanatomical studies to confirm NK3R  
337 protein expression in the mouse hypothalamus, as well as to investigate the potential anatomical  
338 interplay between NK3R and GnRH neurons, as the most plausible kisspeptin-independent  
339 mechanism. Thus,  $WT_{OVX}$ ,  $WT_{OVX+E_2}$ ,  $Kiss1 KO$  and  $Kiss1 KO_{+E_2}$  (n=5/group) were perfused  
340 following standard protocols and the brains were collected for immunohistochemical (IHC)  
341 analyses, as described in detail below. Based on IHC results, a stereotaxic injection approach  
342 was used to specifically activate NK3R in the POA (at the level of the MS), or ARC or MeA and  
343 monitor LH responses in the peripheral circulation of anesthetized  $WT_{OVX+E_2}$  and  $Kiss1 KO_{+E_2}$   
344 females. Unilateral injections were performed as described below on  $WT_{OVX+E_2}$ , and  $Kiss1 KO_{+E_2}$   
345 (n=5/group) which received 600 pmol of senktide diluted in 1  $\mu$ l saline (0.9% NaCl) in the POA or  
346 ARC or MeA. An additional control group was added to the MeA injected cohort which consisted  
347 of  $Kiss1 KO$  mice without  $E_2$  treatment (n=5), which according to results from experiment 1 should  
348 not lead to an increase in LH after senktide administration. Blood samples were collected before

349 (0) and then every 15 min for 45 min after senktide administration. The first blood sample (15 min  
350 post administration) was taken with the needle still in place. Lastly, animals were decapitated and  
351 the brains collected, frozen on dry ice and stored at -80°C for injection site confirmation (see  
352 below).

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

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

363 ***Gonadectomy and sex steroid replacement***

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

372 ***Kiss1 KO GnRH priming***

373 To exclude the possibility that absence in gonadotropin responses to the various stimuli in

374 hypogonadal *Kiss1* KO mice may result from inadequate pituitary responsiveness to GnRH, which  
375 has been previously described in animals with a defective *Kiss1* signaling system (Roa et al.,  
376 2008) *Kiss1* KO mice were subjected to a protocol of GnRH priming during 2 days prior testing,  
377 as has been previously described (Garcia-Galiano et al., 2012). In this protocol, each mouse  
378 received five successive ip boluses of a low dose of GnRH (0.15 µg/each), with the following  
379 schedule: at 10:00 h, 17:00 h, and 23:50 h on the first day; at 0800 and 1600 on the second day  
380 with neuroendocrine tests being conducted on the third day (Garcia-Galiano et al., 2012). WT  
381 mice injected with saline vehicle, following the same protocol, served as controls.

### 382 ***Intracerebroventricular (ICV) Injections***

383 ICV injections were performed following preciously published procedures (Navarro et al.,  
384 2015). Briefly, 2-3 days before the experiment, mice were anesthetized with isoflurane and a small  
385 hole was bored in the skull 1 mm lateral and 0.5 mm posterior to bregma with a Hamilton syringe  
386 attached to a 27-gauge needle fitted with polyethylene tubing, leaving 2.0 mm of the needle tip  
387 exposed. Once the initial hole was made, all subsequent injections were made at the same site.  
388 On the day of ICV injection experiments, mice were anesthetized with isoflurane for a total of 5-  
389 10 min, during which time 5 µl of solution were slowly and continuously injected into the lateral  
390 ventricle. The needle remained inserted for approximately 30 sec after the injection to minimize  
391 backflow up the needle track. Mice typically recovered from the anesthesia within 3 min after the  
392 injection.

### 393 ***Stereotaxic injections***

394 Mice were deeply anaesthetized with isoflurane and placed into a stereotaxic apparatus  
395 (Kopf Instruments, Model 940). After exposing the skull via incision, a small hole was drilled for  
396 injection at the appropriate AP and ML coordinates. A syringe (Hamilton, 5 µL, Model 175 RN  
397 SYR, 32 ga, Cat. No.80016) was lowered into the brain at the appropriate DV coordinates.  
398 Coordinates relative to bregma were as follows: anteroposterior (AP) -1.6 mm, mediolateral (ML)



399  $\pm 0.25$  mm and dorsoventral (DV)  $-5.85$  mm for the ARC, AP  $+0.6$  mm, ML  $\pm 0.25$  mm, DV  $-5.15$   
400 mm for the POA and AP  $-1.9$  mm, ML  $\pm 2.0$  mm, DV  $-4.9$  mm for the MeA. Injection sites were  
401 chosen based on the Paxinos Brain Atlas, and confirmed with India Ink (Fisher Scientific, Cat. No.  
402 NC9903975) trial injections. Each infusion was slowly delivered over 2 min (500 nl/min), the  
403 needle was left in place for an additional 5 min (for AAV injections) and 15 min (for senktide  
404 administrations) and then slowly withdrawn to minimize backflow. Animals received 0.3 mg/kg  
405 buprunex (subcutaneous) during surgery and 24 h later for analgesia and were allowed a 3-week  
406 recovery before onset of experiments.

#### 407 ***Blood Samples and LH measurements***

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

#### 414 **Immunohistochemistry**

##### 415 *Tissue preparation*

416 Animals were terminally anesthetized with a ketamine/xylazine in saline (0.9% NaCl)  
417 cocktail and transcardially perfused with 0.1 M phosphate-buffer (0.1M PB) followed by 4%  
418 paraformaldehyde diluted in 0.1M PB (PFA; Electron Microscopy Sciences). Brains were  
419 removed, stored in the same fixative for 4 hours and then transferred into sucrose solution  
420 [Thermo Fisher Scientific; 20% sucrose in 0.1 M PB containing 0.01% sodium azide (Sigma-  
421 Aldrich)] at  $4^{\circ}\text{C}$ . After sucrose infiltration tissue was cut into 30  $\mu$ m coronal sections using a  
422 freezing stage microtome (Fisher HM440E). The tissue sections were separated into two groups  
423 of three parallel series (90  $\mu$ m 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°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<sub>2</sub>O<sub>2</sub> (10 min; in PBS) and PBS<sup>+</sup> [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*<sup>Cre/+</sup> and *Kiss1*<sup>Cre/Cre</sup> 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<sub>(OVX)</sub>, WT<sub>(OVX+E2)</sub>, *Kiss1 KO* and *Kiss1 KO*<sub>+E2</sub> groups to determine whether E<sub>2</sub>  
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<sub>2</sub>O<sub>2</sub> 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

474 with mutations in the NKB receptor and previously described and validated [NK3R<sup>-/-</sup> mice (True  
475 et al., 2015)]

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

## 480 **Microscopy and image analysis**

### 481 *Validation of senktide injection site.*

482 The locations of POA, ARC and MeA injection sites were investigated in sections cut at  
483 20 µm thickness using a cryostat (Fisher, HM505E). Every other section was collected around  
484 the injection site, mounted on microscope slides air-dried and cover slipped with Vectashield  
485 HardSet Mounting Medium (Vector Laboratories, Burlingame, CA). Only animals with accurate  
486 and restricted injection sites were included in the analysis.

### 487 *Validation of chemogenetic activation of ARC KNDy or MeA Kiss1 neurons.*

488 Sections from animals injected with AAV vectors encoding hM3Dq:mCherry in the ARC or  
489 MeA were examined, and the location of mCherry expression was confirmed in GFP positive  
490 neurons. In both cases, quantification of GFP and GFP/mCherry positive neurons in all areas was  
491 carried out in a subpopulation of animals (n=4/group) with images taken at x20 magnification from  
492 2 representative sections per animal.

### 493 *NK3R/GnRH anatomical relationship*

494 The anatomical relationship between NK3R/GnRH (throughout the hypothalamus),  
495 mCherry/GnRH (in the ARC) and NK3R/NKB (in the MeA) was examined in sections 90 µm apart,  
496 from each mouse. In addition, comparisons of NK3R cell numbers between WT<sub>OVX</sub>, WT<sub>OVX+E2</sub>,  
497 *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<sub>2</sub> on protein expression in  
499 these areas. Counts were averaged per mouse, per brain area. A digital camera (CoolSnap EZ,  
500 Photometrics<sup>TM</sup>, Canada) attached to a microscope (Nikon Eclipse 90i), with the appropriate  
501 excitation for DyLight 488 (green fluorescence) 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  $\pm$  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).

520

521

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## 526 **Author contributions**

527 CF and VMN conceived and designed the research. CF, SL and AMJV conducted  
528 experiments. SLP and RDP generated and provided the *Kiss1<sup>Cre</sup>* mice and validated the *Kiss1*  
529 *KO* model used in the study. CF, SL and VMN contributed to data analysis. CF and VMN wrote  
530 the manuscript, and all authors contributed to manuscript editing.

## 531 **Figure Legends**

532 **Figure 1.** LH levels in male (**A and C**) and female (**B and D**) adult WT and *Kiss1* KO mice, from  
533 blood samples collected before (basal) and 25 min after ICV injection of 600 pmol senktide (an  
534 NK3R-specific agonist). (**A, B**) LH levels in WT male and female mice GNX (WT<sub>ORX</sub> and WT<sub>OVX</sub>,  
535 respectively) and studied in parallel to hypogonadal *Kiss1* KO littermates (n=10/group). (**C, D**) LH  
536 levels in WT and *Kiss1* KO male and female mice with restored levels of sex steroids (WT<sub>ORX+T</sub>,  
537 WT<sub>OVX+E<sub>2</sub></sub>, *Kiss1* KO<sub>+T</sub>, *Kiss1* KO<sub>+E<sub>2</sub></sub>; n=10/group). Paired *t*-test \*\*\*\**P*<0.0001, \*\**P*<0.007 compared  
538 to basal levels of LH. T=testosterone, E<sub>2</sub>=estradiol.

539 **Figure 2. (A)** Schematic representation of the site of injection of an AAV encoding a Cre-  
540 dependent hM3Dq DREADD tagged to mCherry. (**B**) Representative photomicrograph of a  
541 coronal brain section stained for GFP (green), mCherry (red) and merged GFP and mCherry  
542 immunoreactivity in the ARC of a *Kiss1* KO female mouse >3 weeks after hM3Dq:mCherry  
543 injection (Scale bar 50 μm). Mean ± SEM LH responses and area under the curve (AUC) to an  
544 injection of saline (grey line-empty bar) or CNO (green line-green bar) of hM3Dq-injected

545 *Kiss1*<sup>Cre/+</sup> (C) and *Kiss1*<sup>Cre/Cre</sup> (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 WT<sub>ovx</sub> 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<sub>2</sub>-supplemented WT and *Kiss1* KO animals [WT<sub>OVX+E2</sub> and *Kiss1* KO<sub>+E2</sub>,  
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<sub>OVX+E2</sub> (blue line-blue bar) and *Kiss1* KO<sub>+E2</sub> (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<sub>OVX</sub> (left panel) and WT<sub>OVX+E2</sub> (right panel) or *Kiss1* KO (left panel) and  
563 *Kiss1* KO<sub>+E2</sub> (right panel). (C) Mean ± SEM number of NK3R-immunoreactive cells per 30 μm  
564 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  
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*<sup>Cre/+</sup> (G) and  
571 *Kiss1*<sup>Cre/Cre</sup> (KO; H) female mice (n=5/group). opt: optic tract. \**P*<0.025, \*\**P*<0.0014.

572

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## 1 **Supplemental Materials and Methods**

2

### 3 ***Quantitative real-time RT PCR***

4 We aimed to confirm the lack of *Kiss1* expression in the POA and MBH of *Kiss1* KO mice. WT  
5 (OVX; n=3) and *Kiss1* KO (n=5) female mice were killed, brains were exposed and the POA  
6 and MBH was extracted and immediately frozen in dry ice and stored at -80 C. Total RNA  
7 from both areas was isolated using TRIzol reagent (Invitrogen) followed by  
8 chloroform/isopropanol extraction. RNA was quantified using NanoDrop 2000  
9 spectrophotometer (Thermo Scientific) and one microgram of RNA was reverse transcribed  
10 using Superscript III cDNA synthesis kit (Invitrogen). Quantitative real-time PCR assays were  
11 performed in triplicates of each sample on an ABI Prism 7000 sequence detection system,  
12 and analyzed using ABI Prism 7000 SDS software (Applied Biosystems). The cycling  
13 conditions were as follows: 2 min incubation at 50°C, 10 min incubation at 95°C (hot start), 40  
14 amplification cycles (95°C for 15 s, 60°C for 1 min, and 45 s at 75°C, with fluorescence  
15 detection at the end of cycles 3 – 40), followed by melting curve of the amplified products  
16 obtained by ramped increase of the temperature from 55 to 95°C to confirm the presence of  
17 single amplification product per reaction. The primers used are listed in Table 1. The data  
18 were normalized using L19 primers as an internal control. *Kiss1* expression was detected  
19 using primers: F- CTCTGTGTCGCCACCTATGC R – TTCCCAGGCATTAACGAGTTC.  
20 Values were normalized with housekeeping gene *Rpl19*.

### 21 ***Immunohistochemistry for mCherry and GnRH***

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

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

37

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

43

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

50

51 **Supplemental Figure 3.** Representative merged images of mCherry (red) and GnRH (green)  
52 immunoreactivity in the ARC of *Kiss1*<sup>Cre/+</sup> (**A**) and *Kiss1*<sup>Cre/Cre</sup> (**B**). Right panels are higher  
53 magnifications (Scale bar 20 μm) of boxed areas from left panels (Scale bar 50 μm).

54

55 **Supplemental Figure 4.** Representative photomicrographs depicting dual label detection of  
56 NK3R (green) and GnRH (red) in the ARC of WT<sub>(OVX+E2)</sub> and *Kiss1* KO<sub>+E2</sub> animals. Scale bar:  
57 100  $\mu$ m. Note the lack of NK3R staining as opposed to the absence of E<sub>2</sub> in the ARC (Figure  
58 3).

59  
60 **Supplemental Figure 5.** Representative photomicrographs of a coronal section stained for  
61 NK3R (green), NKB (red) and merged NK3R and NKB immunoreactivity in the amygdala of  
62 (A) WT<sub>(OVX+E2)</sub> and NKB (green) of (B) WT<sub>(OVX)</sub> female mouse. Scale bar: 150  $\mu$ m (C) Enlarged  
63 images depicting NKB fibers (presumably from the CeA) in close contact to NK3R-  
64 immunoreactive cell bodies in a WT<sub>OVX+E2</sub> (left panel) and *Kiss1* KO<sub>+E2</sub> (right panel). Scale bar:  
65 20  $\mu$ m. opt: optic tract, MeA: medial amygdala, CeA: central amygdala.

66

## 67 **References**

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72



Figure 1

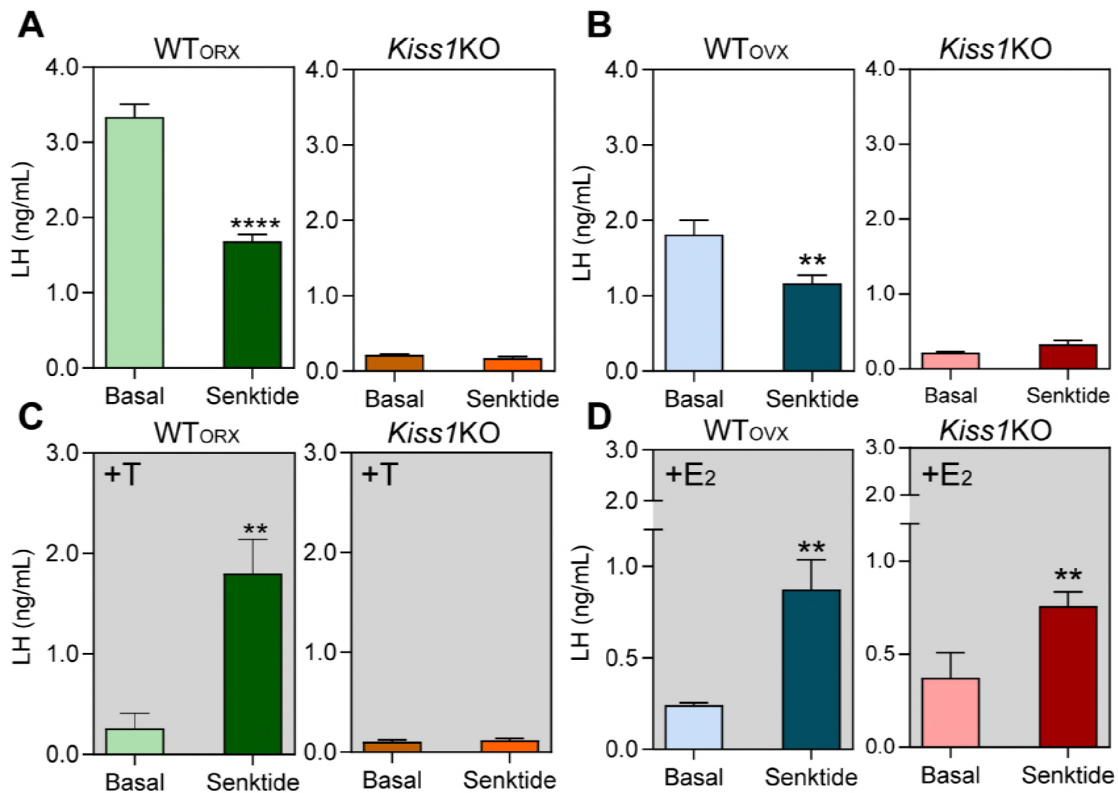


Figure 2

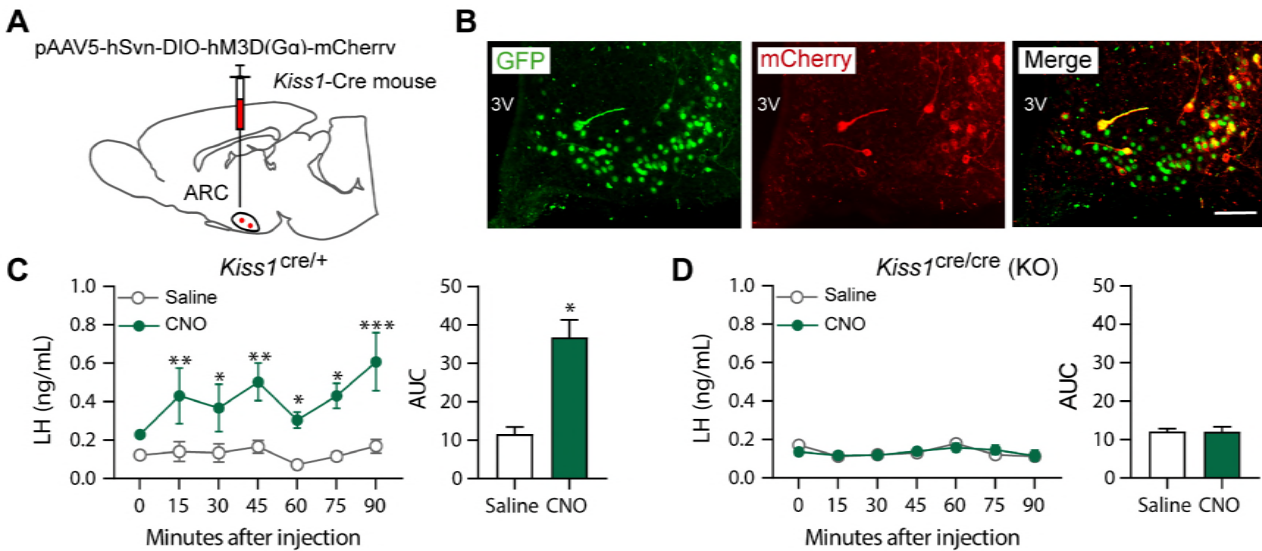


Figure 3

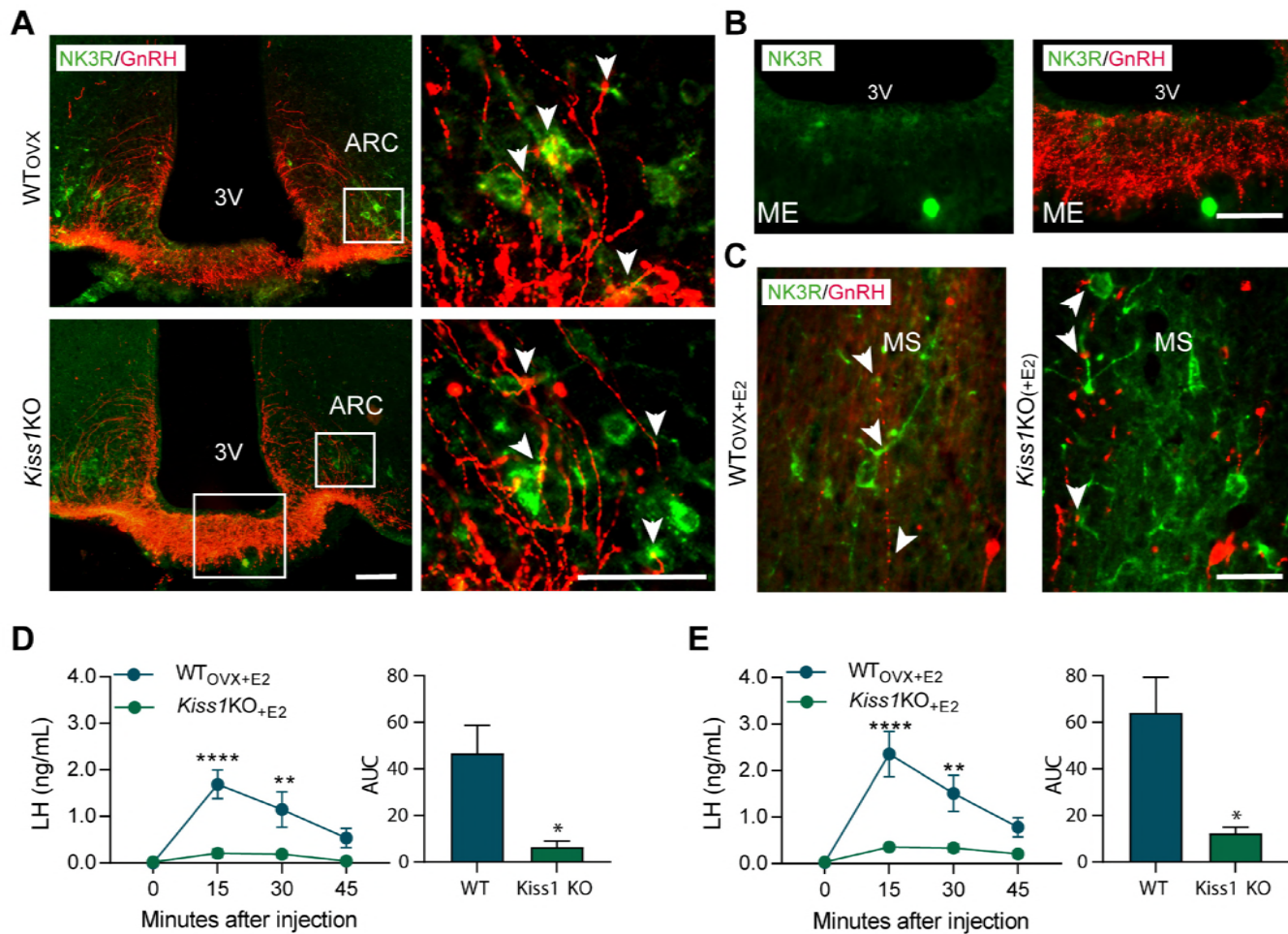
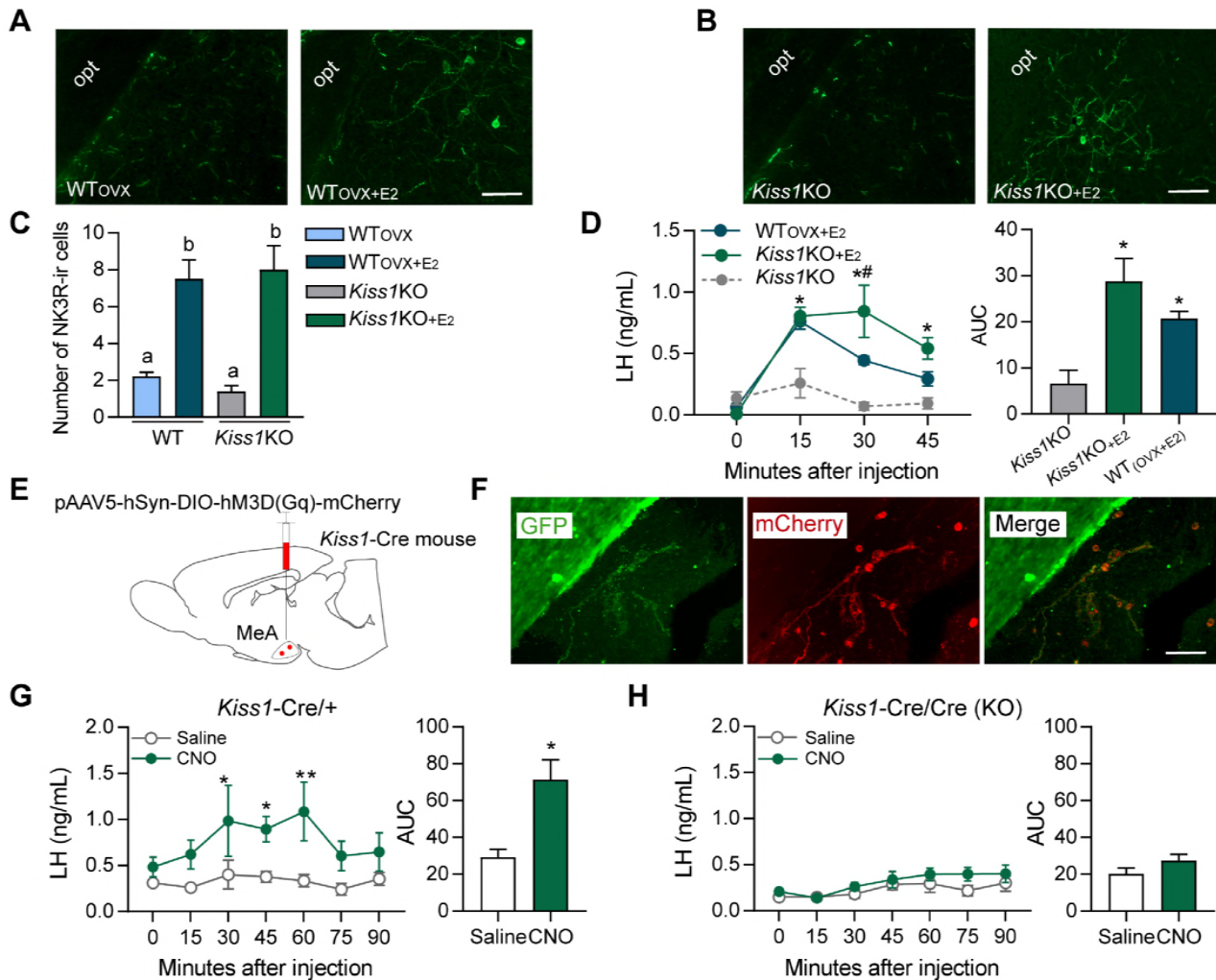
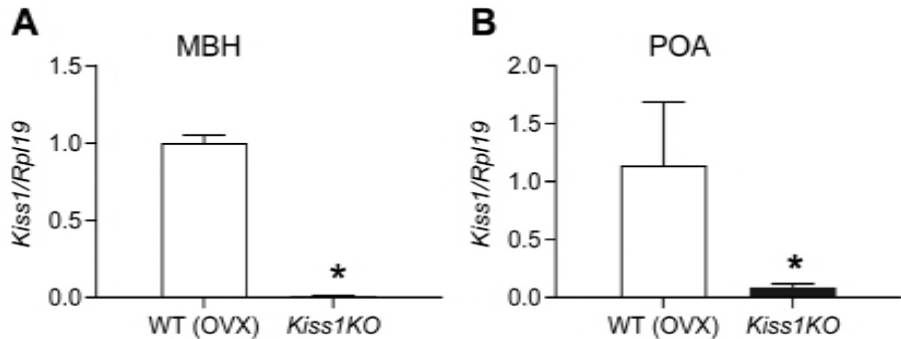


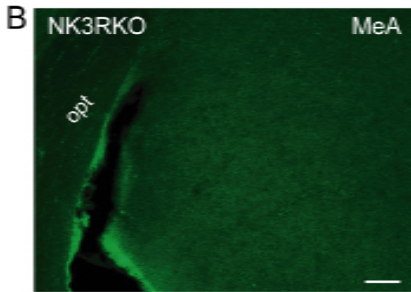
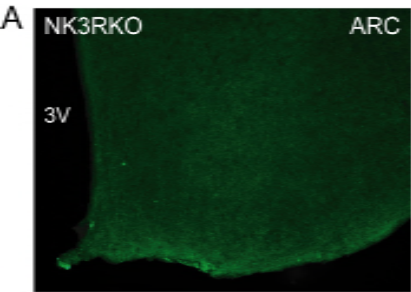
Figure 4



Supplemental Figure 1



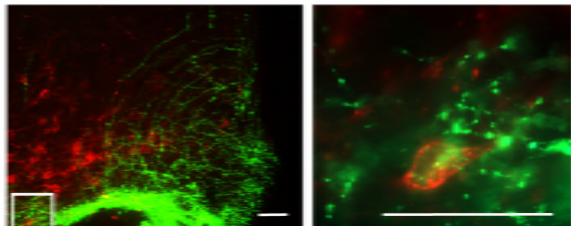
# Supplemental Figure 2



Supplemental Figure 3

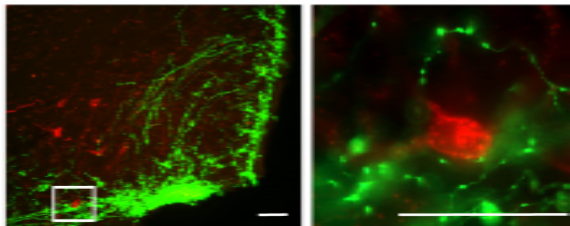
**A**

mCherry/GnRH *Kiss1*<sup>cre/+</sup>

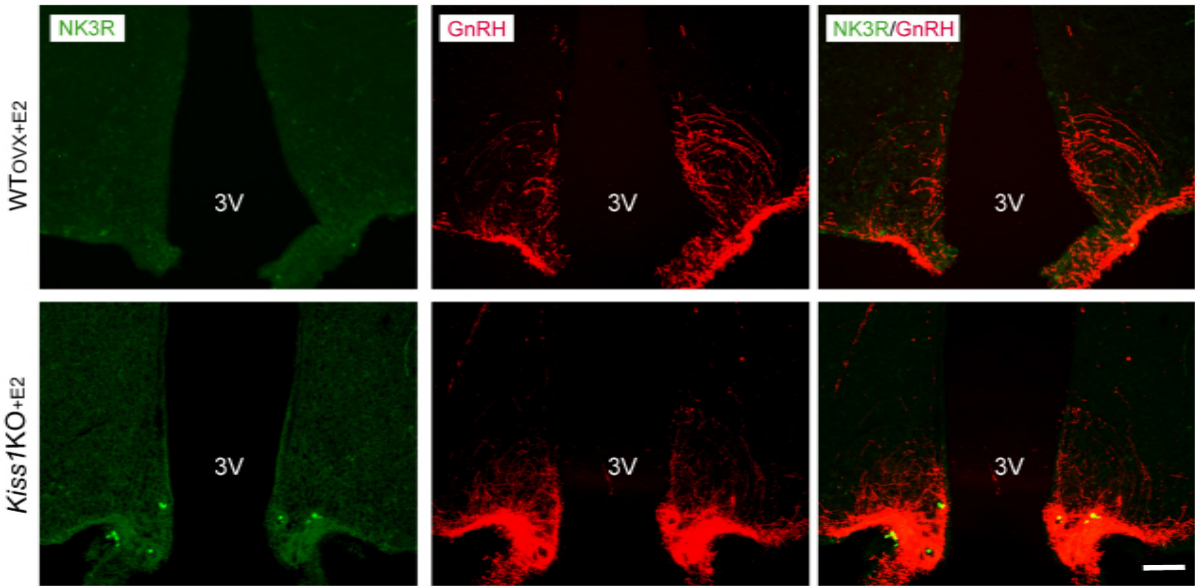


**B**

mCherry/GnRH *Kiss1*<sup>cre/cre</sup> (KO)

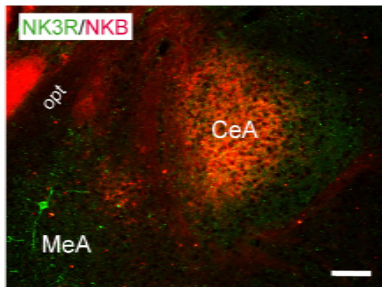
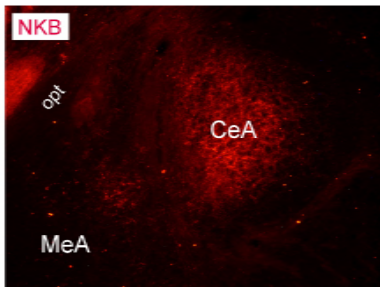
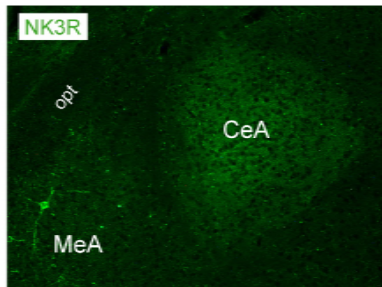


Supplemental Figure 4

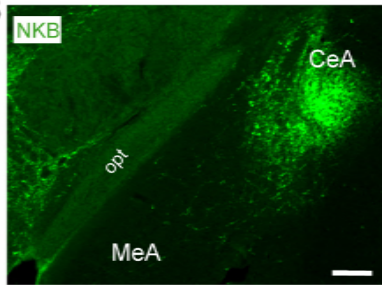




**A**



**B**



**C**

