A comprehensive chemotyping and gonadal regulation of seven kisspeptinergic neuronal populations in the mouse brain

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Abstract

Background: Kisspeptinergic signaling is crucial for reproductive regulation, but its broader roles in brain function are less understood. This study investigates the distribution and chemotyping of kisspeptin-expressing neurons across multiple brain regions in mice.

Methods: Using dual and multiplex RNAscope, we analyzed *Kiss1* mRNA expression in seven kisspeptinergic populations across the four hypothalamic and three extra-hypothalamic nuclei. Co-expression with other neuropeptides, excitatory and inhibitory neurotransmitter markers, and sex steroid receptors was assessed. *Kiss1* expression was also examined following gonadectomy to evaluate androgen receptor involvement in kisspeptin regulation.

Results: Seven distinct kisspeptin neuronal chemotypes were characterized in mouse brain. A novel kisspeptin-expressing group in the ventral premammillary nucleus, co-expressing vesicular glutamate transporter 2, pituitary adenylate cyclase-activating polypeptide, and neurotensin mRNAs was identified. *Kiss1* mRNA was also localized in both somatic and dendritic compartments of hypothalamic neurons. Altered *Kiss1* expression following gonadectomy suggests a role for androgen receptors in regulating kisspeptin signaling.

Conclusion: This study provides a detailed chemoanatomical map of kisspeptin-expressing neurons, highlighting their functional diversity and providing a context for non-endocrine role(s) of kisspeptin in the brain. The discovery of a new kisspeptin-expressing group and gonadectomy induced changes in *Kiss1* expression patterns suggest broader roles for kisspeptin in brain functions beyond reproduction.

Key words: *dual in situ hybridization (DISH), neuropeptides, Esr1, Ar, gonadectomy, vesicular amino-acid transporters*

1. Introduction

The kisspeptins, which include KP-54, KP-14, KP-13, and KP-10 [1, 2], are neuropeptides first identified in human melanoma cells as products of the metastasis-suppressor gene *Kiss1* [3]. Kisspeptin (KP) and its receptor (GPR54 or KISS1R) [4], are important for cell signaling within the hypothalamus in regulating reproductive function through modulation of GnRH secretion [5-14]. KP neurons are also located within extrahypothalamic circuits, notably in the amygdala, where emotion and cognition are integrated and functionally linked to reproduction and reproductive behaviors [15-19].

Early *in situ* hybridization (ISH) assays and immunohistochemistry in mice revealed five kisspeptin mRNA (*Kiss1*)-expressing neuronal clusters in the mouse brain [5, 6, 20-25], with the most prominent in the arcuate nucleus (Arc), and in the rostroperiventricular region of the third ventricle (RP3V) a region which includes the periventricular nucleus (PeN), the anteroventral periventricular nucleus (AVPV), and some scattered cells in the anterodorsal preoptic area. The three other regions that have been reported to express *Kiss1,* albeit at lower levels, are the medial amygdala, the bed nucleus of the stria terminalis and the dorsomedial hypothalamus [26]. Although both sexes express *Kiss1* mRNA in all five areas, expression in the AVPV has been reported to be sexually dimorphic, with the expression of *Kiss1* mRNA and KP greater in females than in males [5, 22].

The regulation of kisspeptin expression and release is complex and may be brain region-specific, both during development, and as a result of epigenetic and environmental factors such as sex steroids, gonadal status, stress and feeding state [27-32]. Gonadal status and kisspeptin signaling exert mutual complex regulation of the hypothalamic-pituitary-gonadal (HPG) axis.

The R3PV kisspeptin neurons play a key role in the positive feedback mechanism of estrogen, which is critical for the preovulatory surge of luteinizing hormone (LH) and gonadotropin-releasing hormone (GnRH). This surge, essential for ovulation, is triggered by elevated estrogen levels during the follicular phase of the menstrual cycle. Notably, these neurons express estrogen receptor alpha (ERα), and their activation enhances Kiss1 expression, which subsequently stimulates GnRH neurons to release GnRH in a pulsatile manner [33-35]. In contrast, ARC kisspeptin neurons are integral to the negative feedback regulation of GnRH secretion. High estrogen levels suppress Kiss1 expression in these neurons, dampening the activation of GnRH neurons and reducing LH release the frequency and amplitude of GnRH pulses, which helps maintain the regular pulsatile release of LH which is necessary for normal reproductive function [33, 36-38].

KP neurons co-express several other neuropeptides that contribute to the dynamics of GnRH regulation [13]. KP^{Arc} neurons co-express kisspeptin, neurokinin B, and dynorphin (and are designated as "KNDy" neurons for this reason) [39-41] as well as galanin [42]. Neurokinin B and dynorphin seem to act autosynaptically on kisspeptin neurons in the Arc to synchronize and shape the pulsatile secretion of kisspeptin and corresponding release of GnRH from fibers in the median eminence [13].

KP *per se* plays an excitatory neurotransmitter role acting upon transient receptor potential canonical (TRPC) and potassium channels expressed in a subpopulation of GnRH neurons [43, 44]. TRPC channels are a subfamily of nonselective cation channels that are part of the TRP superfamily [45, 46]. They are calcium-permeable and receptor-operated, and are activated by the phospholipase C (PLC) signaling pathway [44]. Effects on GnRH excitability of KP neurons co-expressing gamma-aminobutyric acid (GABA) or glutamate have been studied mainly in the KP^{RP3V} and KP^{Arc} populations, respectively [47] [48]. KP direct excitatory effects on GnRH neurons can be potently modulated by GABA, via GABAA and GABAB receptors, and by glutamate, in a spatial-temporal and steroid-dependent manner [12-14, 49]. Kisspeptins can also activate non-GnRH neurons that synapse upon GnRH neurons, exerting indirect effects on GnRH neuronal responses by modulating fast synaptic transmission through GABA and glutamate [50] [51].
As a result of this complex regulation, many circuit-level questions remain about the pathways , direct and indirect, through which KP cooperates with amino acid and neuropeptide co-transmitters to control GnRH neurons and the hypothalamogonadal (HPG) axis, as well as non-HPG neuronal activity controlling various behaviors. Thus, it is essential that chemotyping of KP neurons throughout the brain be achieved at a high level of granularity.

The objectives of our study were first, to characterize kisspeptinergic neuronal populations, based on their molecular signatures and distribution throughout the mouse brain using dual and multiplex RNAscope method and second, to assess the impact of gonadectomy on each of these KP neuronal populations in both male and female wild type mice. Seven distinct *Kiss1*-expressing populations and their sensitivity to gonadectomy are described in detail. The chemoanatomical mapping of KP neurons throughout the brain described here aims to lay the foundation for a better understanding of how KP neurons modulate sexual reproduction and potentially other reproductive behavior-related sensory-emotion-cognitive functions through their peptidergic, glutamatergic and GABAergic synaptic connections.

2. Materials and methods

2.1. Animals

C57BL/6 mice (N=24, male = 12, female = 12) were obtained from the local animal vivarium and housed three per cage under controlled temperature and illumination (12h/12h) with water and food *ad-libitum*. Mice subjected to gonadectomy were singly housed for recovery for one week after surgery and then returned with their cage mates. All animal procedures were approved by the local research ethics supervision committees (license UNAM: CIEFM-079-2020, and NIMH-IRP ACUC).

2.2. Gonadectomy (GNDx)

2.2.1 Mouse ovariectomy (OVX) A dorsal approach to remove the ovaries was used [52]. Briefly, 6 animals were anaesthetized with ketamine 75 mg/kg + xylazine 16 mg/kg i.p. under anesthesia, animals were placed in ventral recumbency and a 3 cm-square region centered over the third lumbar vertebra shaved.

The shaved area was disinfected with Povidone and 70% alcohol, and a sterile fenestrate drape was placed over it. A single midline dorsal incision approximately 1 cm long was made in the skin at the level of the bottom of the rib cage. The incision was moved laterally to cut the right and left abdominal walls. After cutting the wall (obliques and transverse abdominal muscles), blunt dissection through the body wall with mosquito hemostats was performed. Forceps were gently and carefully introduced to retrieve and exteriorize the corresponding ovary. The ovary, ovarian vessels, and ligament were ligated with 4-0 synthetic absorbable sutures and cut. The wall muscle layers incision was closed with a 4-0 synthetic, monofilament, absorbable suture. The skin was closed using a monofilament non-absorbable suture in an interrupted pattern. Surgical glue (Vetbond™ or Dermabond™) was used to assure secure closure. After surgery the animals were monitored and after recovery from anesthesia returned to single cages within the home colony room. Mice were examined for signs of infection or swelling twice per day for one week. If present, non-absorbable suture material was removed from skin closures within fourteen days. Animals received a NSAID (ketoprofen 5 mg/kg SID) via subcutaneous injection, once daily, for analgesia. The NSAID will be continued for a total of 3 days.

2.2.2 Mouse orchiectomy (ORCH)

For the excision of the testicles, we followed an abdominal approach [53] to minimize incidence of testicular herniation. Six animals were anesthetized with ketamine 75 mg/kg + xylazine 16mg/kg i.p. and placed on the operating table on their back. The ventral caudal abdomen were shaved, then scrubbed with three alternating cycles of betadine scrub, 60-70% isopropyl or ethyl alcohol rinse, and finally a dilute betadine solution. All solutions were kept near body temperature and minimal volumes were used to prevent heat loss. When loss of reflexes was confirmed, a 1 cm transverse incision was made through the skin and then through the muscular wall of the ventral caudal abdomen. Each testicular fat pad was identified and followed to find the testicle. A single ligature 4-0 synthetic absorbable suture around the vas deferens and blood vessels was made, prior to excision and gentle removal of the testis. Any residual bleeding was stopped before releasing the fat pad back into the abdomen. The body wall was closed with absorbable suture in a simple interrupted pattern. The abdominal skin was closed using a monofilament non-absorbable suture in an interrupted pattern. Surgical glue (Vetbond™ or Dermabond™) was used to assure secure closure.

2.2.3 Validation of Gonadectomy (GNX) Success:

The success of gonadectomy procedures was ensured through multiple steps. For females, the absence of vaginal proestrus smears was confirmed by performing vaginal cytology during consecutive days post-surgery. Proestrus was identified by the presence of numerous nucleated epithelial cells, and its absence in ovariectomized mice (characterized by abundant leukocytes and the absence of nucleated epithelial cells) validated the success of ovariectomy. For males, physical confirmation of the removal of testes was made during surgery and through careful post-surgical examination. All procedures were conducted by experienced personnel, and recovery was monitored for signs of infection or complication

2.3 Assessment of reproductive cycle

Vaginal smears were performed during consecutive days in control mice to collect brains of mice in proestrous stage, the evaluation of ovariectomized mice confirmed the success of the ovariectomy procedure. Briefly, mice were restrained by. hand, and the vulva was exposed. Using a pipette with a 100 µm tip, a volume of 10 µl of isotonic saline was used to wash the vaginal cavity, then the liquid was deposited in a clean glass slide and left to dry, slides were counterstained with crystal violet stain (0.1%) and observed under a light microscope at 10X and 20X objectives. During proestrus leucocytes are absent and numerous oval nucleated epithelial cells are observed. In the ovariectomized mice abundant leukocytes and some cornified epithelial cells were observed.

2.4 RNAscope single and dual in situ hybridization (DISH) procedure

Detailed methods are described elsewhere [54-56] Briefly, after 7-8 weeks of gonadectomy male and female mice were deeply anaesthetized with sodium pentobarbital (Sedalpharma, México, 100 mg/kg b.w., i.p.) and decapitated using a small animal guillotine (Kent Scientific Corp.), brains from control and gonadectomized mice were removed and rapidly frozen in pulverized dry ice. The fresh-frozen brains were sectioned in sagittal and coronal planes with a Leica CM-1520 cryostat and mounted on positively charged Superfrost Plus microscope slides (Cat. 22-037-246, Fisher Scientific, Pittsburgh, PA).

To determine the expression level of KP mRNA male and female mice and after gonadectomy, we performed a mapping of *Kiss1* expression in coronal and sagittal sectioned brains using the highly sensitive RNAscope SinglePlex (brown) ISH method. Sections were prepared at a thickness of 12 µm, and every fourth section was processed. For the sagittal sections tissue was collected from the midline to lateral 3.5mm according to Paxinos mouse brain atlas [57]. For coronal sectioned brains, tissue was collected between bregma 1.0 mm to -3.5 mm and from bregma -5 mm to -8.5 mm. This systematic sampling ensured an even distribution of analyzed sections across the medio-lateral and rostro-caudal axis. Four mice were processed for each experimental condition. And sections were pretreated accordingly to the manufacturer instructions, incubated with the *Kiss1* (channel 1 probe), followed by the amplification an detection steps.

To evaluate the co-expression of other mRNAs in the seven populations of identified *Kiss1* expressing neurons, we used RNAscope duplex and multiplex ISH methods. Continuous sagittal 12 µm sections were collected from two mice per condition spanning the seven regions of interest. Sections were pretreated, incubated with combinations of the *Kiss1* (channel 1 probe) and other (channel 2 probes) against *Slc32a1, Slc17a6, Ar, Esr1, Tac2 or Pdyn* mRNAs. For the newly described PMv region, a section was processed in a multiplex experiment using Kiss1-C1, Nts-C2 and Adcyap1-C3 probes. After incubation, sections were processed for amplification and detection of the mRNAs, according to the manufacturer instructions.

The probes used to detect the mRNAs were designed by Advanced Cell Diagnostics (https://acdbio.com/rnascope-25-hd-duplex-assay) for mice brains we used the following: kisspeptin (Mm-*Kiss1*, channel 1 probe, Cat No. 500141); for the double in-situ (DISH) experiments the channel 2 probes were designed for detection of the mRNAs of VGAT (Mm-Slc32a1, Cat No. 319191-C2); for VGLUT2 (Mm-Slc17a6, Cat No. 428871-C2); for neurokinin B (Mm-Tac2, Cat No. 446391-C2); for dynorphin (Mm-Pdyn, Cat No. 318771-C2), for estrogen receptor alpha (Mm-Esr1, Cat No. 496221-C2), and for androgen receptor (Mm-Ar, Cat No. 316991-C2). For the Multiplex ISH probes for PACAP (*Adcyap1,* Cat No. 405911-C2) and neurotensin (Nts, Cat No. 420441-C3) were used.

2.5 Imaging and analysis

To quantify the expression level of Kiss1 in the SinglePlex (brown) ISH reacted sections. Photomicrographs were taken at 40x and in a defined region of interest (ROI) chosen based on consistent anatomical landmarks across samples and experimental groups, at least five photomicrographs were taken per animal, per region. In each photomicrograph a region of 200 μm^2 was delimited for analysis. In the ARC and R3PV The *Kiss1* labelling was very intense, with the mRNA puncta clustering densely, covering the soma and extending into dendrites in some neurons. Moreover, the density of KP neurons varied greatly in the mediolateral, anteroposterior and dorsoventral axis of R3PV population of neurons, and in the mediolateral and dorsomedial axis of ARC. For these reasons, the fraction of *Kiss1*+ neurons with dendritic expression in relation to the total number of *Kiss1*+ neurons that were observed in the 200 μm^2 delimited area was quantified. For the other regions (DMH, PMv, Shy, MeApd, and NTS), the labelling intensity of *Kiss1* was much lower, with clearly separated dots surrounding the nucleus of the cells, in this case the average number of *Kiss1* mRNA puncta per cell within those ROIs were quantified, and the average number of the observations from five mice per group were reported.

To evaluate the fraction of *Kiss1* neurons that co-express the mRNAs for VGLUT2 (*Slc17a6*), VGAT (*Slc31a1*), neurokinin-B (*Tac-2*), dynorphin (*Pdyn*), estrogen receptor alpha (*Esr1*), androgen receptor (*Ar*), neurotensin (*Nts*) and PACAP (*Adcyap1*), we quantified the fraction of neurons that co-expressed at least 5 dots of each mRNA evaluated, the analysis was made in 200 μm^2 areas within the ROI, chosen based on consistent anatomical landmarks across samples and experimental groups. Since in the extrahypothalamic regions the density of KP neurons is low, the spatial distribution is heterogeneous, the density of neurons can vary with sex and gonadal status (MeA and Shy), and there can be some individual differences in the staining intensity. Moreover, with the number of animals we used and the separation between sections that was required to have sequential slices reacted against six different mRNAs, we did not found a clear effect of gonadectomy on the fraction of Kiss1 co-expression with other mRNAs nor a sex difference. Thus, here we report a semiquantitative value that we consider provides evidence of the potential co-expression of *Kiss1* mRNA in each of the seven described KP populations and reflects that these population of cells expressions level can be dynamic, and influenced by internal status and external

stimuli. The semiquantitative scores reported here are: (n.o.) for not observed, (+) for <25%, (++) for 26-50%, (+++) for 51-75% and (++++) for >75%.

2.6 Statistical analysis

Statistical analysis was performed using GraphPad Prism 10 Software. Data were assessed for normality using D'Agostino-Pearson test. To compare the fraction of *Kiss1*-positive neurons with dendritic mRNA expression, or the average number of *Kiss1* positive mRNA puncta btween groups for each of these regions, a Two-way ANOVA with sex and treatment as factors was was used.. A Tuckey Post-hoc test was used for pairwise comparisons following a significant ANOVA. Differences were considered statistically significant at $p < 0.05$. Different letters above the bars in the graphs were used to indicate significant differences between groups.

3. Results

3.1 Anatomical localization and molecular signatures of seven populations of *Kiss1*-expressing neurons

We used RNAscope *in situ* hybridization (ISH) technology_which provides enhanced sensitivity, low background and high-resolution spatial information within a neuroanatomical context, in hematoxylin counter-stained permanently mounted sections. This allowed us to examine chemotypic information under the light microscope with single cell resolution. We also studied mRNA co-expression using dual ISH (DISH) with the RNAscope duplex method to determine the GABAergic (assessing the co-expression of *Kiss1* with *Slc32a1*, the mRNA transcript encoding the vesicular GABA transporter) and glutamatergic (co-expression of *Kiss1* with *Slc17a6*, the mRNA transcript encoding the vesicular glutamate transporter 2) nature of each of the KP cell groups in male and female mice. At a single-cell level, we evaluated the co-expression of *Kiss1* with the mRNAs coding for estrogen receptor alpha (*Esr1)* and androgen receptor (*Ar*), as well as for relevant neuropeptides such as neurokinin B (*Tac2*) and dynorphin (*Pdyn*). In the newly characterized population of the ventral premammillary nucleus, we also assessed the co-expression with PACAP (*Adcyap1*) and neurotensin (*Nts*). Sensitivity of *Kiss1* expression to gonadal steroids was quantitatively assessed by the RNAscope (Brown) ISH method in four conditions: male intact and after 7-8 weeks of orchiectomy (ORX) and female intact, at proestrus, or after 7-8 weeks of ovariectomy (OVX).

3.1.1 Kisspeptin neuronal group in the rostro-periventricular area of the hypothalamus (KP^{RP3V})

The RP3V region (Fig. 1A) hosts one of the two major *Kiss1*-expressing neuronal groups, which has been extensively studied over the past two decades. It encompasses *Kiss1* neurons distributed through the ventromedial preoptic nucleus (VMPO), the anteroventral periventricular nucleus (AVPV), the periventricular nucleus (Pe), and the median preoptic nucleus (MnPO). The density of neurons sharing the same molecular signature in this region is heterogeneous, with a wide, sparse distribution in the antero-

ventral and postero-dorsal axis clearly observed in a parasagittal section (Fig. $1A_1$) and narrow-columnar distribution around the third ventricle at the periventricular nucleus level with sharply decreasing density in the medio-lateral direction, evident in coronal section (Fig. $1A_2$, bottom inset).

RNAscope Brown-kit technology allows the visualization of single RNA molecules within cells. It shows that *Kiss1* RNA is extensively expressed in proximal dendrites, in addition to cell bodies, in KP^{RP3V} neurons (Fig. 1A, panels and insets, yellow arrows). This phenomenon has been interpreted to suggest local dendritic translation of mRNA. Dendritic *Kiss1* expression was observed in both male and female brain, weaker in male than in female.

The RNAscope dual in-situ hybridization (DISH) method allows evaluation of the co-expression of separate mRNAs within single cells (Fig. $1A_3$). This *Kiss1* neuronal population co-expresses *Slc32a1* (mRNA for VGAT) and not *Slc17a6* (mRNA for VGLUT2). Some *Kiss1* neurons express *Pdyn* (mRNA for dynorphin) but are negative for *Tac2* (mRNA for neurokinin). The KP^{RP3V} group strongly co-expressed *Esr1* and *Ar* (mRNAs encoding the estrogen receptor alpha and androgen receptor respectively, double blue/red arrows indicate co-expression), suggesting that the KP neurons in the KP^{RP3V} regions are sensitive to estrogen and androgen acting via ERα and AR.

3.1.2 Kisspeptin neuronal group in the arcuate hypothalamic area (KP^{Arc})

The hypothalamic arcuate nucleus hosts a second extensively studied hypothalamic KP-expressing group of neurons (Fig. 1B). These also showed both somatic and dendritic expression of *Kiss1* (Fig. $1B_1$, yellow arrows). Using DISH (Fig. $1B_3$), we confirmed the co-expression of mRNAs for the vesicular glutamate transporter 2 (*Slc17a6)*, neuropeptides neurokinin B (*Tac2*) and dynorphin (*Pdyn*), as previously reported [39, 41, 58]. *Kiss1*-positive neurons strongly expressed estrogen receptor alpha (*Esr1)* and weakly expressed androgen receptor (*Ar)* mRNAs. KP^{Arc} neurons did not co-express *Slc32a1,* the mRNA encoding VGAT.

3.1.3 Kisspeptin neuronal group in the dorso-medial hypothalamic area (KP^{DMH})

Kiss1-expressing neurons were found in the dorso-medial hypothalamic region (DMH, Fig 1C),beginning immediately caudal to the paraventricular nucleus posterior division and extending to the lateral posterior hypothalamic region (LHp). In the dorso-ventral dimension KP cells are dispersed between the dorsal hypothalamic area and the ventromedial nucleus. The cells of this group had low *Kiss1* expression, averaging five mRNA puncta per cell (Fig. $1C_1$). Analysis of DISH experiments (Fig. $1C_2$ and Fig 3) showed that about half of the KP^{DMH} cells express *Slc17a6*, and about a quarter express *Slc32a1* and *Ar*. Some KP^{DMH} cells expressed *Ar and Pdyn*, but not *Esr1* or *Tac2* mRNAs.

3.1.4 Kisspeptin neuronal group in the ventral premammilary nucleus (KP^{PMv})

A newly described population of cells expressing KP mRNA (*Kiss1*) was identified caudally to the Arc, in the ventral premammillary nucleus (PMv, Fig 1D). These neurons had a low expression of KP (average

5-6 puncta per cell), and were densely packed and homogeneously distributed throughout the PMv (Fig. $1D_1$). Using dual and multiplex in situ hybridization experiments (Fig $1D_2$) we showed that these *Kiss1*+ neurons co-express the vesicular glutamate transporter 2 (*Slc17a6*) mRNA and show a low expression of *Ar* and *Esr1*. They also express the mRNA for PACAP (*Adcyap1*) and neurotensin (*Nts*). No co-expression with *Tac2* or *Pdyn*, mRNAs that are strongly expressed in KP neurons of the adjacent arcuate region, was observed.

3.1.5 *Kiss1*-expressing neuronal populations in amygdala (KP^{MeA})

A small population of *Kiss1*-expressing neurons exists in the medial postero-dorsal nucleus of the amygdala (KP^{MeA}, Fig 1E and E_1), at the same rostrocaudal level as the KP^{PMv} neurons. This extrahypothalamic population has been studied by several groups [17, 19, 59], although less extensively that the Arc and RP3V KP populations. The amygdalar *Kiss1* neurons co-expressed *Slc32a1 (mRNA for VGAT*), with a few expressing VGLUT2 (*Slc17a6*) (Fig. $1E_2$). Some of these neurons co-express dynorphin (*Pdyn*), but not neurokinin B (*Tac2*). KP^{MeA} neurons co-expressed *Ar* and, at a lower level, *Esr1*.

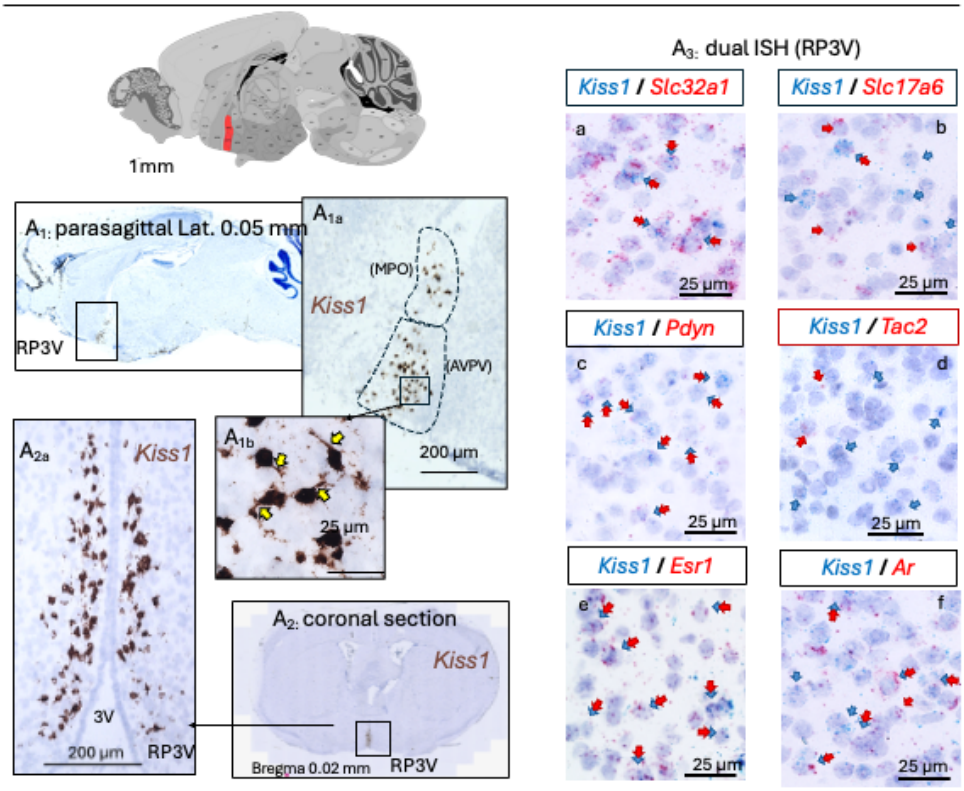
3.1.6 *Kiss1*-expressing neuronal populations in septo-hypothalamic nucleus and surrounding area of the anterior commissure (KP^{SHy})

Dispersed *Kiss1*-expressing neurons with similar molecular signatures as those of the medial amygdala group *(KP^{MeA})* were found surrounding the caudal part of the anterior commissure, spanning the septo-hypothalamic (SHy) area [57], and the stria terminalis (Fig. 1F and F_1), confirming previous reports using IHC [60], ISH and transgenic animals [24, 61, 62]. These neurons co-express *Slc32a1*, as well as *Esr1* and *Ar* (Fig. 1 F_2). No *Slc17a6*, *Pdyn* or *Tac2* were detected in these neurons.

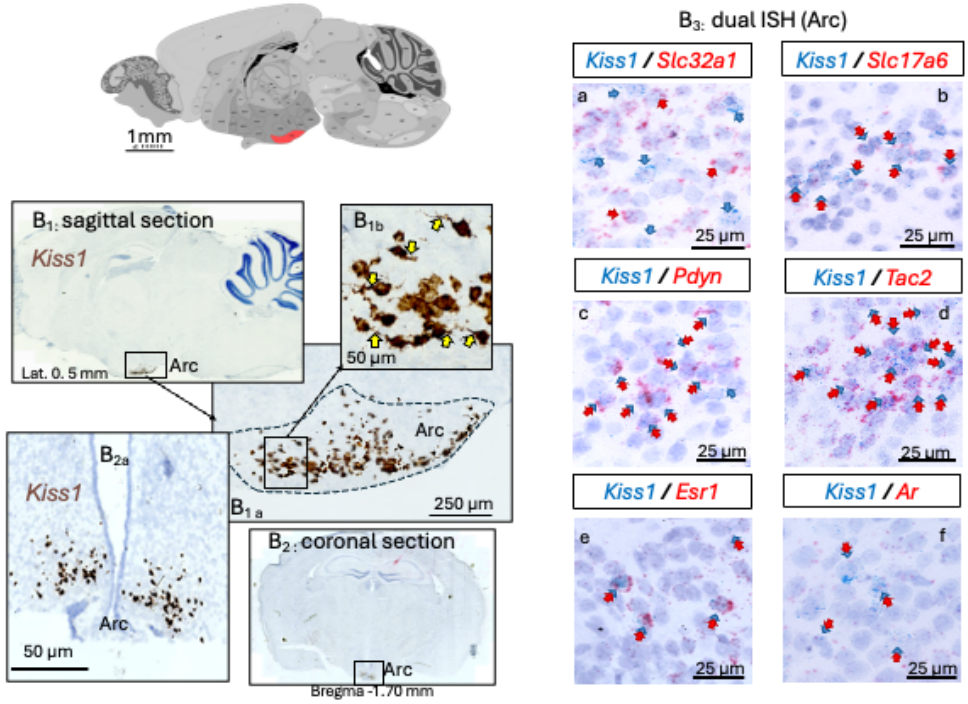
3.1.7 *Kiss1*-expressing neuronal populations in metencephalon

The metencephalic tractus solitarii (NTS) contains the most caudal population of KP neurons reported using kisspeptin immunoreactivity [63, 64]. Here, we show the expression of kisspeptin mRNA (Fig. 1G and G_1) in Sol. Analysis of DISH experiments (Fig $1G_2$ and Fig 3) showed that KP neurons expressed VGLUT2 (*Slc17a6*), with approximately 40% of them expressing *Tac2*, 25% *Esr1,* and most expressing *Ar*. No coexpression of *Pdyn* or *Slc32a1* was observed.

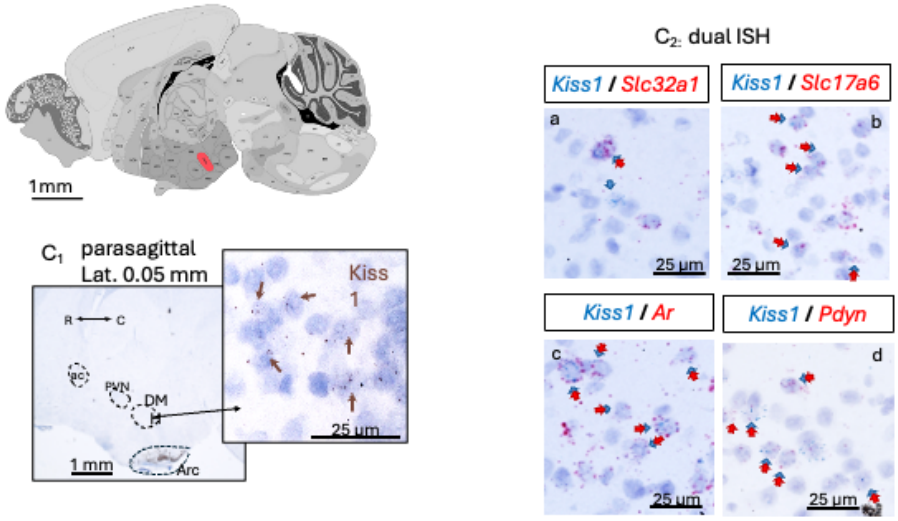
A: RP3V


1mm
A1: parasagittal Lat. 0.05 mm
RP3V
A1a
(MPO)
Kiss1
(AVPV)
200 µm
A1b
25 µm
A2a
Kiss1
3V
200 µm
RP3V
A2: coronal section
Kiss1
Bregma 0.02 mm
RP3V
A3: dual ISH (RP3V)
Kiss1 / Slc32a1
Kiss1 / Slc17a6
Kiss1 / Pdyn
Kiss1 / Tac2
Kiss1 / Esr1
Kiss1 / Ar
a
b
c
d
e
f
25 µm

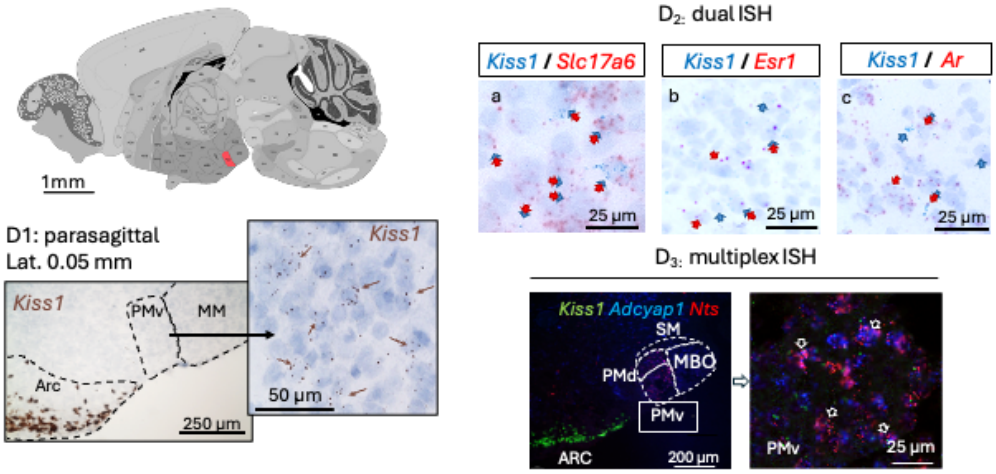
B: Arc


1mm
B1: sagittal section
Kiss1
Lat. 0. 5 mm
Arc
B1b
50 µm
Arc
250 µm
B1a
B2a
Kiss1
Arc
50 µm
B2: coronal section
Arc
Bregma -1.70 mm
B3: dual ISH (Arc)
Kiss1 / Slc32a1
Kiss1 / Slc17a6
Kiss1 / Pdyn
Kiss1 / Tac2
Kiss1 / Esr1
Kiss1 / Ar
a
b
c
d
e
f
25 µm

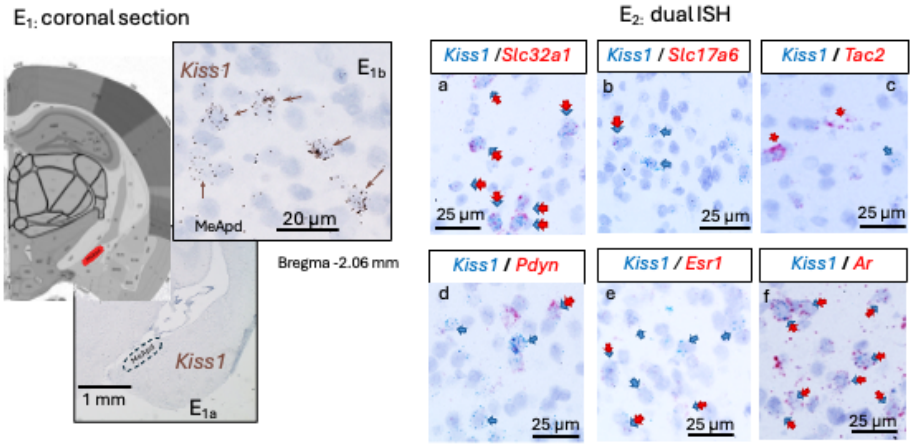
C: DMH



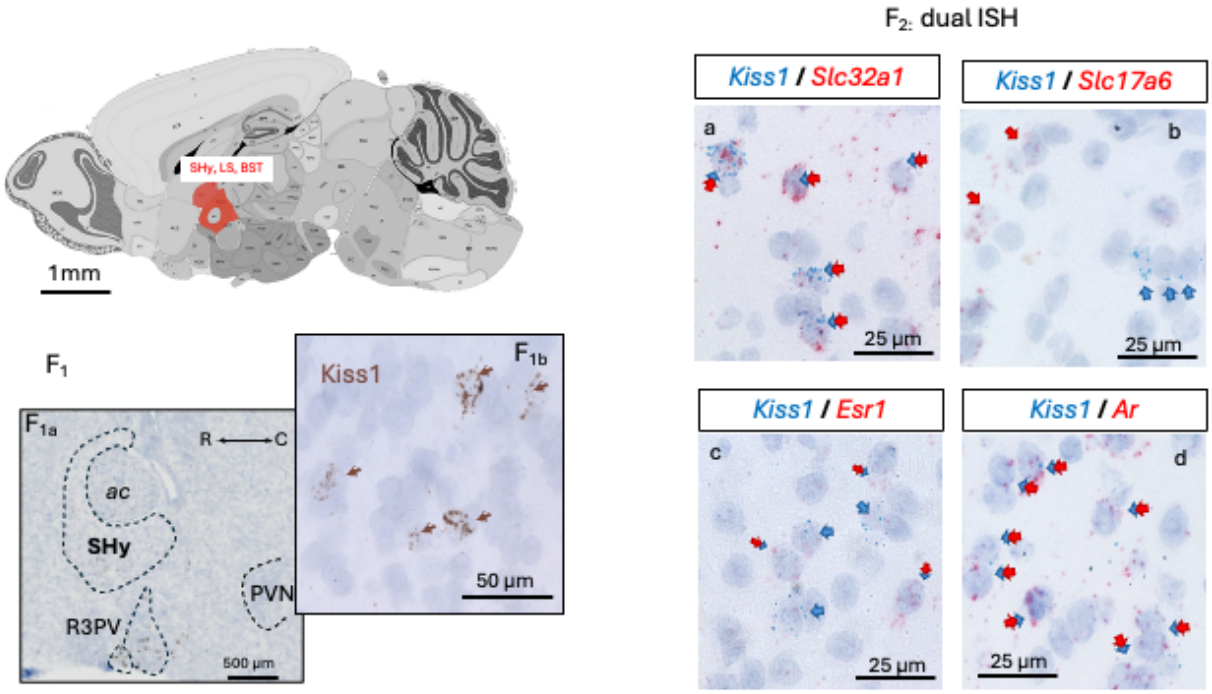
D: PMv



E: MeA



F: SHy (includes dispersed cells in LS and BST)



G: NTS

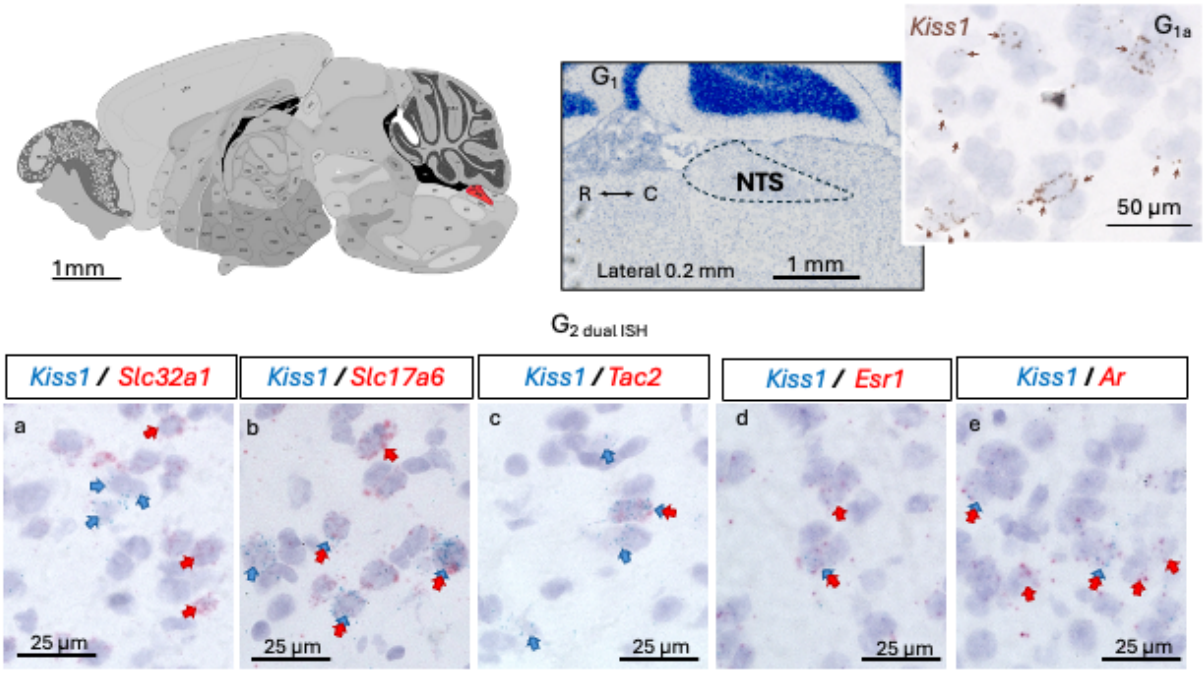


Figure 1: ***In situ hybridization (ISH) characterization of seven Kiss1-expressing neuronal populations in the mouse brain.*** *A: the rostro-periventricular area of the hypothalamus (KP^{RP3V}) shown in a parasagital (A_1 and inset) and in a coronal view (A_2) have a heterogeneous spatial distribution in the rostro-caudal axis and a steep decrease in density in the medial to lateral direction. The yellow arrows in the figures indicate cells with prominent expression of Kiss1 mRNA in the dendritic compartment. A_3 panels show RNAscope duplex reactions that demonstrate the expression of Kiss1 (blue arrows), and other mRNAs (red arrows) coding for the vesicular GABA transporter (Slc32a1), the vesicular glutamate transporter 2 (Slc17a6), the neuropeptides neurokinin B (Tac2), dynorphin (Pdyn), the sex steroid estrogen receptor alpha (Esr1) or the androgen receptor alpha (Ar). Double arrows show that in this region Kiss1 is co-expressed with Slc32a1, Pdyn, Esr1 and Ar. B: Kisspeptin expression in the arcuate nucleus of the hypothalamus (KP^{ARC}). B_1 and B_2 show Kiss1 expressing neurons in the arcuate nucleus some of them with dendritic Kiss1 expression (yellow arrows). B_3 panels show that in this population Kiss1 is colocalized with Slc17a6, Tac2, Pdyn, Esr1 and Ar mRNAs but not with Slc32a1. C: In the dorso-medial hypothalamic area (KP^{DMH}) Kiss1 detected by singleplex RNAscope is shown in a parasagittal section in C_1, Kiss1-expressing neurons in the DMH are indicated by brown arrows in the inset. The mRNA expression levels were low with approximately 5 puncta per cell in both sexes and gonadal conditions. Duplex RNAscope photomicrographs in C_2 show that in this population Kiss1 is colocalized with Slc17a6, Slc32a1, Pdyn and AR. No co-expression with Tac2, or Esr1 mRNAs was observed. D: Kisspeptin mRNA expression in the ventral premammillary nucleus area (KP^{PMv}). Low and high magnification photomicrograph of the PMv, taken from a sagittal section that was processed for Singleplex (C_1) shows Kiss1 epressing neurons scattered in the PMv, the expression levels of Kiss1 was low with approximately 5-6 puncta per cell in both sexes and gonadal conditions. Duplex and multiplex RNAscope reactions show that this newly characterized population of Kiss1 neurons co-express the mRNAs for Slc17a6, Esr1 and AR, PACAP (Adcyap1) and neurotensin (Nts). E: Kisspeptin expression in the medial amygdala (KP^{MeA})***.*** *Panel E_1, low and high magnification photomicrographs of a coronal section showing Kiss1 expressing neurons concentrated in the posterodorsal part of the medial amygdala (MeApd). Duplex RNAscope (E_2) showed that Kiss1 neurons strongly co-expressed Slc32a1 and Ar and to a lower level also expressed Slc17a6, Esr1, and Pdyn. F: Kisspeptin expression in the septo-hypothalamic area (KP^{SHy}). Panel F_1, parasagittal section depicts the SHy area, spanning septal, BNST and hypothalamic regions where scattered low Kiss1-expressing neurons (inset of F_1) were found around the anterior commisure. F_2, photomicrographs taken from SHy in slices treated for RNAscope Duplex. Kiss1 was observed to be colocalized with Slc32a1, Esr1 and AR. No co-expression with Tac2, Pdyn or Slc17a6 mRNAs was observed in this region. G: Kisspeptin expression in the NTS (KP^{NTS}). Singleplex RNAscope (G_1), showed Kiss1 expressing neurons located in the solitary tract nucleus of male and female mice. G_1 and inset, photomicrograph of a sagittal section depicting neurons expressing Kiss1 at low abundance. G_2 panels show by RNAscope Duplex the co-expression of Kiss1 mRNA with Slc17a6, Tac2, Esr1 and AR. No co-expression with Slc32a1 or Pdyn was observed.* Single blue arrows represent *Kiss1* mRNA only and double blue-red arrows represent colocalization of tested mRNAs. The performance of each assay was validated by examining other regions within the same brain slice known to express the

second mRNA, ensuring that the absence of colocalization was not due to technical issues but rather accurately reflected the absence of co-expression in the specific neuronal population.

3.2 Kiss1 expression's vulnerability of the seven populations by gonadectomy

Seven weeks after gonadectomy (GNX), there was an evident reduction in *Kiss1* expression levels in the KP^{RP3V} and an increase in the KP^{Arc} cell populations observed under the microscope. However, given the high sensitivity of the RNAscope assay and the heterogeneous distribution of this population of *Kiss1* neurons, we found that counting cell density in a given section, with a certain number of *Kiss1* labeled dots, cannot objectively reflect the observed reduction/augment. We sought a new method to assess the changes in *Kiss1* expression after short-term GNX, specifically by quantifying the percentage of *Kiss1* positive neurons that expressed mRNA in the dendritic compartment.

The *Kiss1* neurons of female proestrous mice were more strongly labelled, and a significantly higher ($p<0.001$) percentage of them showed dendritic mRNA expression, compared to males (71.2% ± 3.5% in females vs. 27.5% ± 2.6% in males) , see Fig. 2A and A1 *vs*. A3. After gonadectomy there was a reduction in the number of cells expressing dendritic *Kiss1*: OVX (5.1 % ± 1.5%, $p< 0.001$ vs. female intact) and ORX (ORX = 1.7% ± 1.2%, $p< 0.001$ vs. male intact).

In the KP^{Arc} region the quantification of the percentage of KP^{ARC} neurons with somatodendritic *Kiss1*-expression versus total labeled cells (Fig. 2B) showed a significantly higher percentage of dendritic *Kiss1*-expressing neurons in intact male mice (20.4 ±1.24%) compared to female proestrus mice (6.4 ±1.4 %). After gonadectomy, there was a potent upregulation of *Kiss1* dendritic expression in the Arc region in both sexes, with the expression levels in females surpassing that of males (ORX = 69.7 ± 3.86 vs OVX = 79.3 ± 1.02, $p < 0.05$).

Quantification of the average number of *Kiss1* mRNA puncta per cell in the KP^{DMH}, KP^{PMv} and KP^{NTS} showed no differences between male and female mice, or any effect of gonadectomy in males or females (Fig. 2C, 2D, 2G).

In KP^{MeA}, GNX induced a significant decrease in the average number of *Kiss1* mRNA puncta per cell only in male mice (Fig 2E). Stephens and Kauffman [59] reported up-regulation of *Kiss1* in MeA after estrogen treatment of gonadectomized male mice, and aromatase expression is higher in male mice than in females [65, 66]. Androgens may exert direct effects on *Kiss1*-expression via signalling through the highly expressed *Ar* as well as indirectly, through the *Esr1,* after aromatization to estrogen. According to this scenario, it is likely that the sexually dimorphic response to gonadectomy in this population of neurons is because the drop in steroid signalling after gonadectomy, since it is through both *Ar* and *Esr1* in the male, is greater in males than in females.

Similarly to the KP^{MeA} population, only male animals showed a decrease in the expression of *Kiss1* after gonadectomy in KP^{SHy} neurons, from 12.68 ± 1.0 to 7.62 ± 0.62 *Kiss1*-puncta per cell (Fig. 2 F). Interestingly, in both SHy and MeA neurons the existence of an inhibitory control on the expression of *Kiss1* via GABA-B receptors has been demonstrated [24, 62], highlighting the plasticity of these neurons in response to stimuli other than sex steroids.

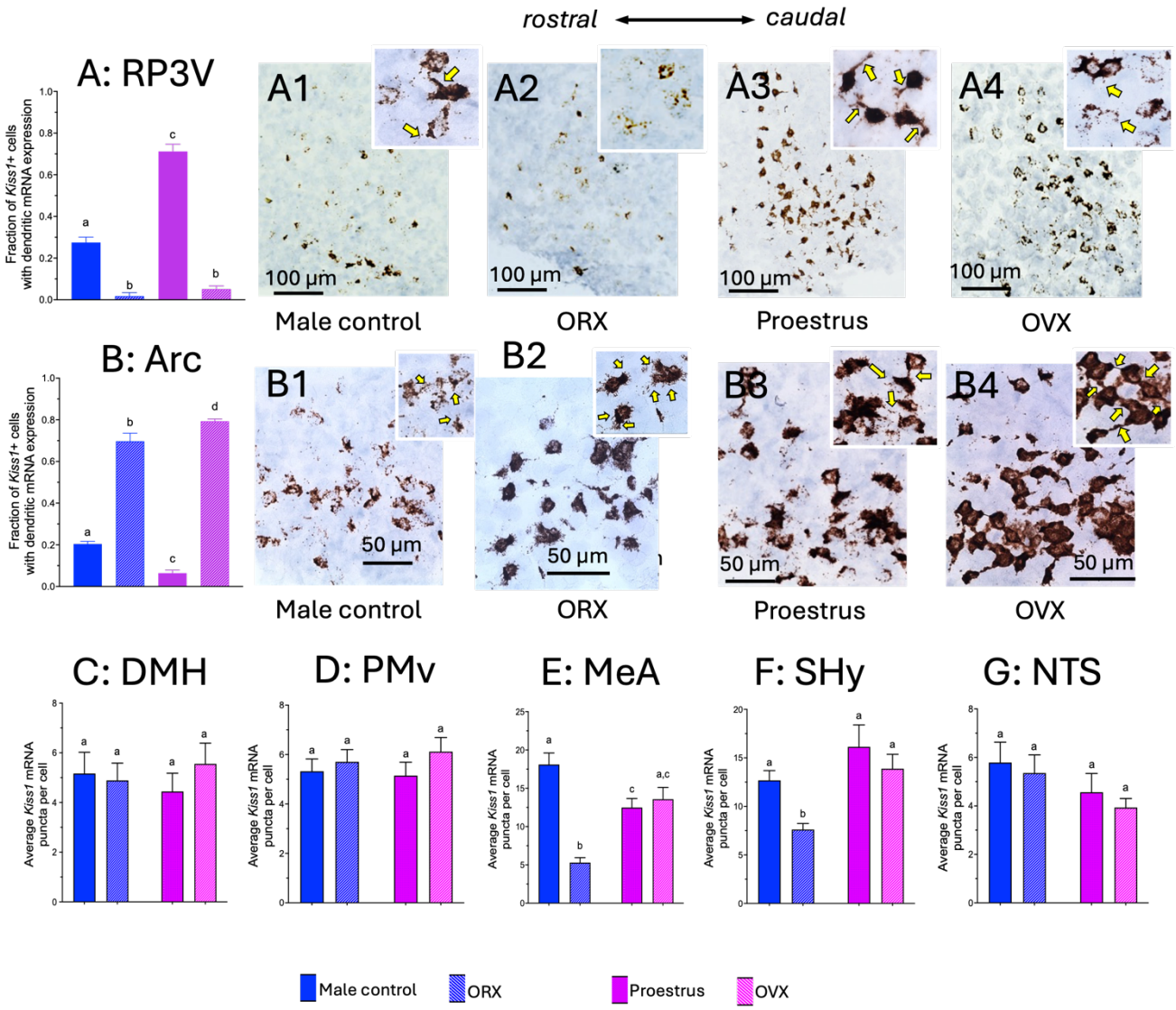
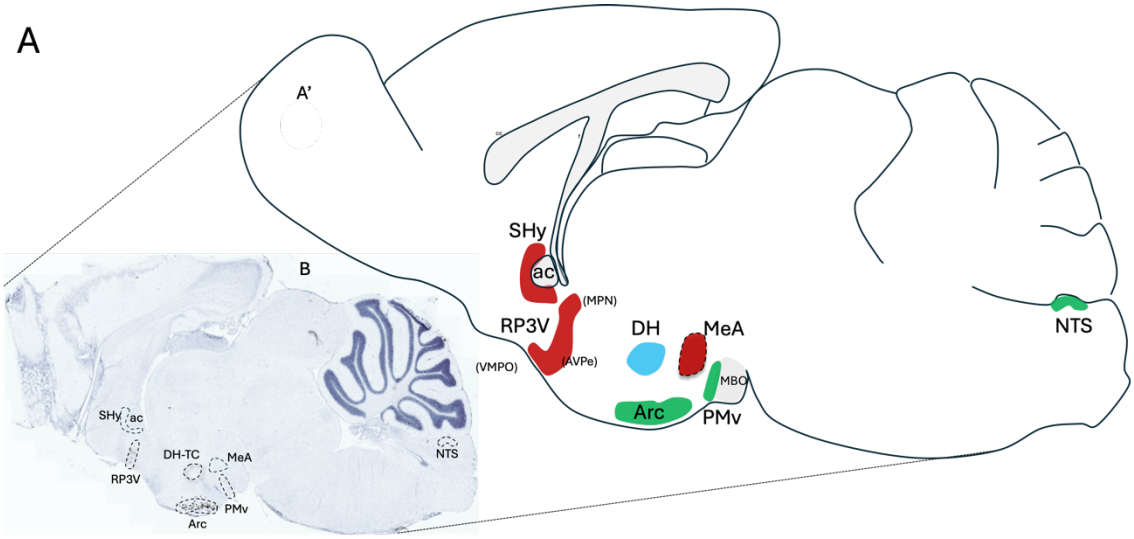


Figure 2: ***Effect of gonadectomy on Kiss1 expression in the seven Kiss1 expressing populations of the mouse brain.*** *A-B: The effects of gonadectomy were quantified in the two major population of Kiss1 expressing neurons, namely the RP3V (panels As) and Arc (panels Bs). The neurons in these regions showed a high cellular expression of Kiss1, and an heterogeneous spatial distribution. These two populations were distinguished by the expression of Kiss1 in the dendritic compartment. Thus we measured the fraction of Kiss1 cells that displayed dendritic Kiss1 expression in relation to the total number of cells expressing Kiss1. This allows a more precise quantification of the effects of gonadectomy, minimizing possible errors derived from the high sensitivity of the test that is able to detect single molecules of mRNA thus detecting cells with insignificant expression and also sampling errors derived from the very heterogeneous spatial distribution of the cells, particularly in the RP3V. Representative photomicrographs of RP3V (A1-A4) and Arc (B1-B4) show low and high magnification of these areas in*

male and female proestrous control mice and after orchidectomy (ORX) and ovariectomy (OVX). Yellow arrows indicate examples of neurons with dendritic expression. In the RP3V the fraction of cells expressing dendritic Kiss1 is higher in proestrous female than in control males, and gonadectomy significantly reduced the dendritic expression in both sexes. In the Arc the male control animals showed a higher fraction of Kiss1 cells with dendritic expression, and after gonadectomy the Kiss1 expression was dramatically increased in both sexes, with the OVX females showing a higher fraction of Kiss1 dendritic expression than the ORX males. Significant ($p < 0.05$) statistical differences between groups are indicated by letters. Bars without shared letters are significantly different. C-F: The expression of Kiss1 in these neuronal populations was much lower than in the RP3V and Arc. F or these regions we quantified the average mRNA puncta per cell. No significant differences in Kiss1 expression levels between males and females, nor any effect after gonadectomy was detected in the hypothalamic KP^{DMH} (panel C) and KP^{PMv} (panel D). For the extrahypothalamic regions, in the medial amygdala (KP^{MeA}, panel E) and the septo-hypothalamic region (KP^{SHy}, panel F) we observed that gonadectomy caused a significant reduction in Kiss1 expression only in the male animals. For the nucleus of tractus solitarius population (KP^{NTS}, panel G), no differences in Kiss1 expression between males and females or after gonadectomy were observed.

An overview of the seven populations of neurons in the mouse brain, with regard to the coexpression of KP mRNA (*Kiss1*) with *Slc32a1* or *Slc17a6,* is represented in the drawing of Fig 3A, in which the regions containing mainly *Kiss1* neurons co-expressing *Slc32a1* are symbolized in red, the regions with *Kiss1* neurons mainly coexpressing *Slc17a6* are represented in green, and the DH region containing both *Slc32a1* and *Slc17a6* Kiss neurons is represented in blue. With the exception of the MeA (indicated with a dotted line border), all the regions are located very close to the midline. Fig 3B, summarizes the molecular signatures of the seven populations of kisspeptinegic neurons, indicating the co-localization of *Kiss1* with *Slc32a1, Slc17a6*, *Pdyn*, *Tac2*, *Esr1* and *Ar*. A semiquantitative report on the co-expression percentages of these mRNAs with *Kiss1* is provided. Also a summary of the effect of orchidectomy (ORX) and ovariectomy (OVX) on *Kiss1* levels for each of the regions described in this study is presented in Fig 3B.



B

	Molecular signature of seven *Kiss1* expressing populations						*Kiss1* expression post-gonadectomy	
	Slc32a1	***Slc17a6***	***Tac2***	***Pdyn***	***Esr1***	***Ar***	♂	♀
RP3V (AVPe-VMPO-Pe-MPN)	++++	+	n.o.	+	++++	+++	↓	↓
MeA	++++	+	n.o.	++	+	+++	↓	No change
SHy	++++	n.o.	n.o.	n.o.	+	+++	↓	No change
DMH-LH	+	++	n.o.	+	n.o.	+	No change	No change
Arc	n.o.	++++	++++	++++	++++	+	↑	↑
PMv	n.o.	+++	n.o.	n.o.	+	+	No change	No change
NTS	n.o.	++++	++	n.o.	+	+++	No change	No change

Fig 3. Molecular signature of seven population of Kiss1 neurons in the brain, *A: parasagittal section and representative drawing of the regions that were observed to host Kiss1 positive neurons. The regions that have mainly GABAergic (Slc32a1) are colored in red, the regions with mainly glutamatergic (Slc17a7) are colored in green, the DM region colored in blue, contains glutamatergic and GABAergic Kiss1 neurons. B: Summary table depicting the co-expression of Kiss1 mRNA with the mRNAs coding for the vesicular GABA transporter (VGAT, Slc32a1); the vesicular glutamate transporter 2 (VGLUT2, Slc17a6); neurokinin B (Tac2), Dynorphin (Pdyn), estrogen receptor alpha (Esr1) and androgen receptor (Ar). The semiquantitative scores represent percentages of coexpresion with Kiss1 neurons: (n.o.) for not observed, (+) for <25%, (++) for 26-50%, (+++) for 51-75% and (++++) for >75% Abbreviations: anterior commissure (ac); anteroventral periventricular nucleus (AVPe); arcuate nucleus (Arc); dorsomedial hypothalamic nucleus (DMH); mammillary bodies (MBO); medial amygdala (MeA), median preoptic nucleus (MPO), nucleus of tractus solitarius (NTS); septo-hypothalamic area (SHy); rostro-periventricular nu. of the third ventricle (RP3V); ventral premammillary nucleus (PMV); ventromedial preoptic area (VMPO).*

4. Discussion

We report here the molecular signatures of seven distinct *Kiss1*-expressing populations and their sensitivity to gonadectomy. For the two major KP hypothalamic groups, namely the rostro-periventricular group (KP^{RP3V}) and the arcuate hypothalamic group (KP^{Arc}), our observations are mainly in accordance with the literature. However, using the highly sensitive "Brown kit" RNAscope method, we observed, and report here for the first time, that in these two populations, besides *Kiss1* mRNA expression in the soma, a high proportion of KP cells express mRNA in *the dendritic compartment*. This phenomenon suggests an ability to localize and translate mRNA in dendrites, allowing neurons to rapidly respond to synaptic signals with spatial precision, thereby enhancing synaptic plasticity [67] [68-70].

For the KP^{RP3V} population we confirmed that KP neurons co-express the *Slc32a1*, *Esr1*, *Ar* and *Pdyn* RNAs. In agreement with most of the previous reports characterizing mRNA expression in this region comparing between sexes and after gonadectomy [20, 23, 71, 72], we observed a higher number of KP neurons in females than in males and a down-regulation of the density of *Kiss1*-expressing neurons in both sexes after gonadectomy. This down-regulation of *Kiss1* expression after gonadectomy was highly significant when evaluating the mRNA expression in the dendritic compartment. Quantification of the percentage of *Kiss1* positive neurons that show dendritic expression avoids a possible selection bias arising from the highly heterogeneous distribution of the neurons in this region.

In the arcuate hypothalamic group (KP^{Arc}), the KP neurons co-express *Slc17a6*, *Esr1*, *Ar*, *Pdyn*, *Tac2*, and have been extensively characterized as "KNDy" neurons [73]. In this study we report that the percentage of *Kiss1*-positive neurons expressing the mRNA in the dendritic compartment was three times higher in males (20.4%) than in proestrous females (6.4%). It has been classically considered that the plasma concentration of estradiol is highest during proestrus [74], however a recent study in mice showed that although in proestrous the estradiol levels do have a peak, it is during diestrous that estradiol concentrations reach their maximum [75]. Thus, in addition to regulation of dendritic Kiss1 mRNA in males compared to females, there may also be differences during the estrous cycle in females as well. Gonadectomy caused a sharp increase in dendritic mRNA labeling within *Kiss1*-expressing cells, to 70% in males and to 79% in females. *Kiss1* dendritic expression in both KP^{RP3V} and KP^{Arc} represents a new aspect of KP cellular physiology, albeit it remains to be correlated with sites of KP expression in, and release from, these neurons.

While the majority of studies using in situ hybridization report a greater number of KP neurons in the RP3V of female rodents, the data on sex differences in mRNA expression within the ARC remain inconclusive. Some studies report no differences in expression [24, 71], while others indicate a higher number of *Kiss1*-expressing neurons in males [20, 23] or in females [72]. These discrepancies may stem from factors such as threshold criteria for *Kiss1*-positivity, the dense neuronal packing within the region, or the varied distribution along the rostrocaudal or mediolateral axes. Focusing on the percentage of *Kiss1* neurons that exhibit dendritic mRNA expression may provide a more consistent and sensitive indicator of

expression changes, capturing dynamic cellular responses with greater precision, in neurons with abundant expression of *Kiss1* mRNA.

A third group of KP neurons in the dorsomedial hypothalamus was identified and characterized at the mRNA level in the present report. Previous studies using immunohistochemistry [22, 63, 76] and Kiss1-CRE mice [77, 78] showed the prescence of neurons in DMH. However, other studies mapping *Kiss1* expression by in situ hybridization failed to show Kiss1 mRNA-positive cells in this region [6, 20]. It has been suggested that this inconsistency between immunohistochemical and in situ hybridization histochemical findings might be explained by the detection of Arg-Phe-related peptide-1 (RFRP-1), rather than kisspeptin, using antiboides cross-reactive for both peptides [36]. However, we confirm here the existence of these dispersed KPergic neurons by RNAscope in situ hybridization. Since the mRNA probe targets a length of 480 base pairs, almost the entire length of the *Kiss1* mRNA, and is intolerant of partial sequence homology for effective hybridization, this technique confirms the Kiss1+ chemotype of these neurons. Here we characterized these KP expressing neurons as capable of co-expressing *Slc17a6* (Vglut2 mRNA), *Slc32a1* (VGAT mRNA) and *Ar* (androgen receptor mRNA) positive. No significant changes were detected after gonadectomy in *Kiss1* mRNA expression in this population.

In the ventral premammillary nucleus (PMv) *Kiss1* mRNA puncta were distributed homogeneously among the densely packed cells, seen clearly in parasagittal sections. The existence of this group of KP neurons was suggested earlier by immunohistochemistry in horse [79] and indirectly by GFP expression in *Kiss1*-GFP transgenic mice [80]. The authors of the study in horse interpreted that these neurons were extended from the Arc neurons, and the results obtained in the transgenic mice study reported expression in several other regions not previously associated with Kiss1 expression, suggesting ectopic GPF expression. Here, we confirm by in-situ hybridization the existence of this population. Furthermore, these neurons do not share the molecular signature of the KP^{Arc} population. Apart from expressing *Kiss1* and *Slc17a6,* the KP^{PMv} neurons do not express *Tac2* nor *Pdyn,* and express *Esr1* only weakly. KP^{PMv} neurons also co-express *Adcyap1* and *Nts*, the mRNAs encoding the pituitary adenylate cyclase-activating polypeptide (PACAP) and neurotensin. Interestingly, the Allen Mouse Brain Connectivity Atlas contains documentation via AAV-aided tracing of genetically targeted PMv Adcyap1-Cre neurons, that these project to the hippocampal CA2 region, a brain region relevant for social behavior [81].

The PMv has been shown to be important in controlling the kisspeptin - GNRH -LH axis, and lesions of this nucleus disrupt both kisspeptin and GnRH expression, and reproductive behaviors [82]. The PMv also links leptin levels to changes in reproductive function [83]. The classical model for this is leptin stimulation of kisspeptin release onto GnRH neurons, however the Arc kisspeptin neurons have not been found to express OB-R (leptin receptor), and thus it has been propossed that leptin indirectly activates the Arc Kisspeptin cells and these in turn activate the GnRH neurons [84]. An interesting alternative possibility is that the KP^{PMv} neurons reported in this study are the mediators that release KP onto the GNRH neurons upon leptin stimulation. It is known that the PMV neurons express the leptin receptor

[85]. Moreover, a recent publication showed that PACAP-glutamatergic neurons that respond to leptin, located in the PMV can regulate the release of GnRH by innervating a subset of Kisspeptin neurons in the Arc and in the RP3V [86].

The extrahypothalamic populations of kisspeptin (KP) neurons may play distinct physiological and behavioral roles beyond those associated with hypothalamic KP neurons. The KP neurons in the MePD have been studied most extensively [17, 24, 60, 62, 76, 87]. This region is involved in processing olfactory stimuli and influencing maing behavior and reproductive functions [88]. KP^{MePD} neurons co-express vesicular GABA transporter (VGAT) and, to a lesser extent, vesicular glutamate transporter 2 (VGLUT2), indicating a predominantly GABAergic but partly glutamatergic phenotype. This neurotransmitter profile, combined with the expression of androgen receptors (AR) and estrogen receptor alpha (ESR1), suggests that MePD KP neurons could influence sociosexual behavior through via sex-hormone signaling pathways. The chemoanatomical markers identified in these MePD cells are congruent with a recent study in *in female mice where RiboTag/RNA-seq was used to demonstrate Pdyn, Ar, and Esr1 expression within Medial amygdala tissue from Kiss1-Cre mice* (Hatcher, Costanza et al. 2023). The male-specific reduction in KP mRNA expression in KP^{MePD} neurons following gonadectomy, reported in this study, supports the role of these neurons in male sexual motivation and reproductive behavior. Noteworthy are recent studies using functional magnetic resonance imaging (fMRI), showing that administering kisspeptin to healthy men produces enhanced activity in the amygdala and interconected regions such as the hippocampus and cingulate cortex which are crucial to emotional regulation, sexual response and bonding [89].

The sixth KP population was widely distributed in the BNST complex, centered in the strio-hypothalamic nucleus, with a spatial continuum spanning septo-strio-diagonal band of Broca ventrally, and is referred to here as KP^{SHy}. This KP population shares a molecular signature with KP^{MePD} neurons, expressing both VGAT and sex steroid receptors (AR and ESR1), suggesting a related functional role in modulating reproductive behaviors. Considering the role of the septum and BNST in emotional processing, stress response, motivation and reward-seeking behavior, as well as their connections with the hypothalamus and other limbic structures [90-93], it is plausible that KP neurons in these regions contribute to modulating these functions. For example, they might influence anxiety levels, motivation related to social interaction, or the integration of emotional states with reproductive behaviors. The similarity in molecular markers and gonadal hormone sensitivity between the MePD and SHy KP neurons highlights a potential role for these neurons in coordinating behaviors that are influenced by both hormonal state and social context, especially in males. We found that in KP^{MePD} and KP^{SHy}, gonadectomy induced a decrease in the average number of *Kiss1* mRNA puncta per cell only in male mice.

A seventh population of extrahypothalamic KP neurons was found in the brain's major sensory hub in the dorsal medulla, the solitary tract nucleus (KP^{NTS}). KP^{NTS} neurons are glutamatergic and co-express *Tac2, AR,* and *ESR1* mRNAs, yet do not exhibit changes in *Kiss1* mRNA expression following gonadectomy in either males or females. Our results confirm by ISH the existence of this group of neurons as reported by immunohistochemistry against metastin [63, 64]. As the NTS is a primary sensory and

autonomic hub involved in processing visceral and sensory information, KP neurons here may play a role in integrating autonomic signals with overall response to stress or energy demands, rather than reproductive behavior per se.

Together, these findings suggest that extrahypothalamic KP neurons, while sharing some common molecular markers, may have region-specific roles that reflect the functional demands of their respective brain areas. KP neurons in the MePD and SHy appear to be closely linked with the modulation of sociosexual and reproductive behaviors, while KP neurons in the NTS might be involved in integrating sensory and autonomic inputs. Further research into the connectivity, physiological responses, and behavioral impact of these KP neurons could provide new insights into the diverse roles of KP signaling in brain function beyond hypothalamic reproductive control.

Systematic examination of steroid hormone receptors in both the well-studied hypothalamic and amygdala KP cell groups, and the less-studied hypothalamic and extrahypothalamic groups provide a molecular explanation for the distinction among KP neuronal populations with respect to changes in KP expression upon gonadectomy. Four patterns of alteration by gonadectomy were observed in this study. Classical down-regulation of *Kiss1* expression by GNX in both sexes in RP3V is consistent with high levels of *Esr1* and *Ar* in RP3V, and positive regulation by sex steroids, in both sexes [73]. Confirmation of up-regulation of KP expression by GNX in arcuate nucleus in both sexes (see above) suggests, given high levels of *Esr1* but only low levels of *Ar* in these neurons, that negative modulation of KP expression may occur via estrogen, implying that aromatase expression in nearby neurons may occur in mammals as it does more prominently in seasonally-breeding animals [94]. The third pattern of alteration by gonadectomy was down-regulation in males but no significant effect in female, in MEA and SHy, again consistent with the relatively high expression of *Ar* but not *Esr1* in these KP cell clusters. The DMH, PMv and NTS showed no significant changes in *Kiss1* expression regulation by GNX in either sex. This finding is consistent with the very low or no expression of either *Esr1* or *Ar* in DMH and PMv, and the lack of *Esr1* in NTS where *Kiss1*-positive cell numbers are low and the expression pattern is sparse. Robust expression of Ar in NTS led us to suspect that this newly characterized KP cell group would be regulated by GNX in male mice, however, this was apparently not the case, although it is feasible that we overlooked some steroid receptor-expressing KP cells in brain stem, given the section intervals used for our survey.

It is intriguing that the two major KP cell groups of hypothalamus, those in RP3V and in Arc, are oppositely regulated by gonadectomy, and are of opposite chemotype (GABAergic and glutamatergic, respectively), and quite consistent with the opposing effects of these two KP populations on reproductive function mediated via GnRH neurons. It has been reported that kisspeptin fibers from these two major KP groups innervate different subpopulations of GnRH neurons, and that there are complex interconnections between these two populations, suggesting a sophisticated feedback loop regulating their activity and ultimatedly impacting GnRH function [95]. A recent study using electrophysiological recordings in combination with optogenetics, showed excitatory and inhibitory projections from the KP^{Arc} and the KP^{RP3V} respectively, converging onto preautonomic paraventricular (PVN) and dorsomedial (DMH)

neurons to control their excitability [96]. How the post-synaptic effects are modulated by co-release of KP, glutamate and GABA from KP^{RP3V} and KP^{Arc} neurons is a question awaiting more detailed investigation.

Significant advance in our understanding of the critical role of kisspeptin in human sexually-related behavior has been provided by clinical studies featuring kisspeptin administration in men and women [84, 89]. However, except for some scattered reports (e.g. [17]), there has been little exploration of kisspeptin projections to areas other than GnRH neurons of the hypothalamus. As pointed out by Liu and Herbison [47], the post-synaptic effects of kisspeptin leading to behavioral effects such as increased sexual responsiveness may be mediated via neuromodulatory rather than direct neurotransmitter mechanisms, and at sites of kisspeptinergic innervation of brain areas involved in both sensorial processing and behavioral state control. Thus, a better understanding of the projection of KP neurons outside of the hypothalamus, arising from both hypothalamic and extrahypothalamic KP cell bodies, is needed. The chemotyping of KP neurons provided here for the mouse provides a context for consideration of how different sets of KP neurons in the mammalian brain work together to orchestrate male and female reproduction, not only via regulation of individual endocrine responses, but by regulation of individual and social behaviors.

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