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

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Abstract

Herein, we present a systematic analysis, using dual and multiplex RNAscope methods, of seven kisspeptinergic neuronal populations, based on their chemotyping and distribution throughout the mouse brain. The co-expression of mRNAs coding for neuropeptides, for excitatory and inhibitory transmitter vesicular transporters, and for sex steroid receptors are described in four hypothalamic and three extra-hypothalamic nuclei. These include a newly characterized kisspeptin-expressing ventral premammillary nucleus cell group co-expressing vesicular glutamate transporter 2, pituitary adenylate cyclase-activating polypeptide and neurotensin mRNAs. Kisspeptin mRNA (*Kiss1)* was observed within both somatic and dendritic compartments at a single-cell level in two hypothalamic sites, a prominent and previously undescribed feature of kisspeptin neurons in these two cell groups. Patterns of altered *Kiss1* expression following gonadectomy among these seven KP populations suggest that androgen receptor signaling may also play a previously unremarked role in gonadal feedback regulation of kisspeptinergic neuronal function. Data from this study provide a chemoanatomical basis for hypothesis generation regarding the functional diversity of kisspeptinergic signaling in hypothalamic and extrahypothalamic brain.

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

## 1. Introduction

The kisspeptins (KP), which include KP-54, KP-14, KP-13, and KP-10 (Kotani et al. 2001; Makri et al. 2008), are neuropeptides first identified as products of the anti-metastasis gene KiSS-1(Lee et al. 1996), now simply called *KiSS-1*. KP and their receptor (GPR54 or *Kiss1r*) that was cloned three years later (Lee et al. 1999), are important for cell signaling within the hypothalamus in regulating reproductive function through modulation of GnRH secretion (Dungan, Clifton, and Steiner 2006; Gottsch et al. 2004; Irwig et al. 2004; Seminara et al. 2003; de Roux et al. 2003; Clarke, Dhillo, and Jayasena 2015; Lehman et al. 2018; Piet et al. 2018; Liu et al. 2021; Liu, Porteous, and Herbison 2022). 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 (Mills et al. 2022; Lopez-Ojeda and Hurley 2022; Pineda et al. 2017; Kim et al. 2011; Gresham et al. 2016).

Early *in situ* hybridization (ISH) assays and immunohistochemistry in mice revealed five kisspeptin mRNA (*Kiss1*)-expressing neuronal clusters in the mouse brain (Smith, Cunningham, et al. 2005; Gottsch et al. 2004; Dungan, Clifton, and Steiner 2006; Lehman et al. 2010; Clarkson and Herbison 2006; Smith, Dungan, et al. 2005; Di Giorgio et al. 2014; Franceschini et al. 2013), 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 remaining regions that have been reported to express *Kiss1* at lower levels are the medial amygdala, the bed nucleus of the stria terminalis and the dorsomedial hypothalamus (Lehman, Hileman, and Goodman 2013). 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 (Dungan, Clifton, and Steiner 2006; Clarkson and Herbison 2006).

The factors that regulate kisspeptin expression and release are complex and not fully understood. These include developmental changes, as well as genetic, epigenetic, and environmental factors, such as sex steroids, gonadal status, stress and feeding state (Hudson and Kauffman 2022; Izzi-Engbeaya and Dhillo 2022; Aerts et al. 2021; Clarke and Dhillo 2016; Pinilla et al. 2012; Huang et al. 2022). Gonadal status and kisspeptin signaling exert mutual complex regulation of the hypothalamic-pituitary-gonadal (HPG) axis. A dual role for estrogen in regulating the two major KP neuronal groups in the hypothalamus has been reported. Estrogen exerts positive feedback on KP neurons in the RP3V region which is crucial for the pre-ovulatory surge of luteinizing hormone (LH) in females necessary for ovulation, whereas it exerts negative feedback on the Arc KP population ($KP^{Arc}$) inhibiting *Kiss1* gene expression and kisspeptin production(Gocz et al. 2022). This inhibition reduces the stimulatory input to GnRH neurons, thereby modulating the frequency and amplitude of GnRH pulses, which helps maintain the regular pulsatile release of LH which is necessary for normal reproductive function(Oakley, Clifton, and Steiner 2009). KP neurons have been reported to co-express several neuropeptides(Liu et al. 2021). For instance, the $KP^{Arc}$ neurons co-express kisspeptin, neurokinin B, and dynorphin (and are designated as "KNDy"

neurons for this reason)(Goodman et al. 2007; Moore et al. 2018; Navarro et al. 2009) as well as galanin(Skrapits et al. 2015). Neurokinin 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 (Liu et al. 2021).

Experimental evidence from early studies established that 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 (Zhang et al. 2008; Liu, Lee, and Herbison 2008). TRPC channels are a subfamily of nonselective cation channels that are part of the TRP superfamily(Wes et al. 1995; Zhu et al. 1995). They are calcium-permeable and receptor-operated, and are activated by the phospholipase C (PLC) signaling pathway(Liu, Lee, and Herbison 2008). Effects on GnRH excitability of KP neurons co-expressing amino acid transmitters gamma-aminobutyric acid (GABA) and glutamate have been studied mainly in the $KP^{RP3V}$ and $KP^{Arc}$ populations, respectively (Liu and Herbison 2016) (Ronnekleiv, Qiu, and Kelly 2022). KP direct excitatory effects on GnRH neurons can be potently modulated by GABA, through differential effects via GABAA and GABAB receptors and glutamate in a spatial-temporal and steroid-dependent manner (Liu et al. 2021; Liu, Porteous, and Herbison 2022; Piet et al. 2018; Iremonger, Porteous, and Herbison 2017). While kisspeptins can directly activate GnRH neurons, they can also activate non-GnRH neurons, that exert indirect effect on the GnRH neuronal responses to kisspeptins by modulating fast synaptic transmission involving ionotropic GABA and glutamate receptors (Pielecka-Fortuna, Chu, and Moenter 2008) (Leon et al. 2016).

Many questions remain unanswered with respect to the pathways (direct/indirect) through which KP cooperates with amino acid and neuropeptide co-transmitters, especially in kisspeptin regulation in circuits beyond the GnRH neurons and the hypothalamogonadal (HPG) axis.

With the availability of duplex and multiplex RNAscope ISH methods, we sought to make a comprehensive chemotyping study of KP cell groups at a single cell level, taking into account the potential co-expression of neuropeptides, amino acid transmitters and sex steroid receptors. 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 of each of these KP neuronal populations in both male and female wild type mice. Seven distinct *Kiss1*-KP populations and their sensitivity to gonadectomy are described in detail. The KP-GABAergic populations are the hypothalamic rostro-periventricular group ($KP^{RP3V}$), the strio-hypothalamic complex with continuation to peri-anterior commissural region ($KP^{SHy}$), and the medial amygdala group ($KP^{MeA}$). The dorso-medial hypothalamic group ($KP^{DMH}$) was observed as weakly co-expressing both *Slc32a1* and *Slc17a6,* mRNAs for VGAT and VGluT2. respectively. The KP-glutamatergic populations are the classical arcuate hypothalamic group ($KP^{Arc}$) and two newly identified populations, the ventro-premammilary nucleus ($KP^{PMv}$) and the nucleus of the solitary tract ($KP^{NTS}$). At the single cell level, *Kiss1* is prominently expressed in both *somatic* and *dendritic compartments* in $KP^{RP3V}$ and $KP^{Arc}$. The seven major KP cell groups were also chemotyped for co-expression with the

neuropeptides neurokinin (*Tac2*) and dynorphyn (*Pdyn*) as well as with the gonadal steroid receptors for estrogen and androgen (*Esr1* and *Ar*), and the effects of gonadectomy on *Kiss1* expression in both male and female mice were analyzed. GNX reduced the dendritic expression of *Kiss1* in $KP^{RP3V}$ where both *Esr1* and *Ar* are expressed, but increased it in $KP^{Arc}$, where *Esr1* is robustly expressed but *Ar* is only weakly expressed, without evident change in *Kiss1*-labeled cell number in either sex, or in either nucleus. In $KP^{MeA}$ and $KP^{BST}$, GNX reduced single cell RNA puncta only in male subjects, suggesting that *Ar* signaling plays a critical role in KP expression in these nuclei in male mice. GNX did not exert a measurable effect on *Kiss1* expression in $KP^{DMH}$, $KP^{PMv}$, or $KP^{NTS}$. 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. Results

### 2.1 Anatomical localization and molecular signatures of seven populations of *Kiss1*-expressing neuronal populations

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 estrogen receptor alpha (*Esr1)* and androgen receptor (*Ar*), as well as relevant co-expressed neuropeptides such as neurokinin (*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*). *Kiss1* expression level was quantitatively assessed in four conditions: male control and after 7-8 weeks of orchiectomy (ORX) and female control at proestrus or after 7-8 weeks of ovariectomy (OVX).

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

The RP3V (Fig. 1A and A') hosts one of the two major *Kiss1*-expressing neuronal groups, which has been extensively studied over the past two decades. Using RNAscope Brown-kit technology, that allows the visualization of a single RNA molecule per cell, we have observed that *Kiss1* RNA is extensively expressed in proximal dendrites, in addition to cell bodies, in this neuronal population (Fig. 1A, panels and insets, yellow arrows), indicating the likely translation of *Kiss1* mRNA locally in dendrites of $KP^{RP3V}$ neurons. Dendritic *Kiss1* expression was observed in both male and female brain, although the expression

in male mice is weaker than in females. This phenomenon has been interpreted as an ability to localize and translate mRNA in dendrites, potentially allowing neurons to rapidly respond to synaptic signals with spatial precision, hence enhancing synaptic plasticity (Bramham and Wells 2007) (Holt, Martin, and Schuman 2019). The RNAscope dual in-situ hybridization (DISH) method allowed us to evaluate the co-expression of two mRNAs at single cell level (Fig. 1A, lower panels). 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. This *Kiss1* neuronal population co-expresses *Slc32a1* (mRNA for VGAT) and do not express *Slc17a6* (mRNA for VGLUT2). Some *Kiss1* neurons express *Pdyn* (mRNA for dynorphin) but are negative for *Tac2* (mRNA for neurokinin).

The RP3V region (Fig. 1 A and A') encompasses *Kiss1* neurons distributed through the following hypothalamic nuclei: the ventromedial preoptic nucleus (VMPO), the anteroventral periventricular nucleus (AVPV), the periventricular nucleus (Pe), and the median preoptic nucleus (MnPO). The density of the neurons sharing the same molecular signature in this space-continuum is heterogeneous, with a wide sparse distribution in the antero-ventral and postero-dorsal axis clearly observed in a parasagittal section (Fig. 1A') and narrow-columnar distribution around the third ventricle at periventricular nucleus level with the density decreasing sharply in the medio-lateral direction evident in a coronal section (Fig. 1A, bottom inset).

### 2.1.2 Kisspeptin neuronal group in the arcuate hypothalamic area ($KP^{Arc}$)

The hypothalamic arcuate nucelus hosts the other major extensively studied hypothalamic KP-expressing population. The neurons in this region also showed somatic and dendritic expression of *Kiss1* (Fig. 1B, yellow arrows). With the DISH method, we confirmed the co-expression of mRNAs for the vesicular glutamate transporter 2 (*Slc17a6)*, neuropeptides neurokinin (*Tac2*) and dynorphin (*Pdyn*), (Fig. 1B, bottom panels), which are previously reported (Moore, Coolen, and Lehman 2019; Goodman et al. 2007; Navarro et al. 2009). *Kiss1*-positive neurons strongly expressed estrogen receptor alpha (*Esr1)* and weakly expressed androgen receptor (*Ar)* mRNA*s*. $KP^{Arc}$ neurons did not co-express *Slc32a1,* the mRNA encoding VGAT.

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

*Kiss1*-expressing neurons were found in the rostro-caudal dimension 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). About half of the $KP^{DMH}$ cells express *Slc17a6*, and about a quarter express *Slc32a1* and *Ar*. No expression of *Tac2* or *Pdyn* was observed in $KP^{DMH}$ neurons.

### 2.1.4 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). These neurons show a low expression of KP (average 5-6 puncta per cell), and are densely packed and homogeneously distributed throughout the PMv (Fig. 1D). Using dual and multiplex in situ hybridization experiments we showed that these *Kiss1*+ neurons co-express the mRNA for PACAP (*Adcyap1*) and neurotensin (*Nts*) (Fig. 1D′, fluorescent microphotographs). They also express the vesicular glutamate transporter 2 (*Slc17a6*) mRNA and show a low expression of *Ar* and *Esr1*.

### 2.1.5 *Kiss1*-expressing neuronal populations in amygdala ($KP^{MeA}$)

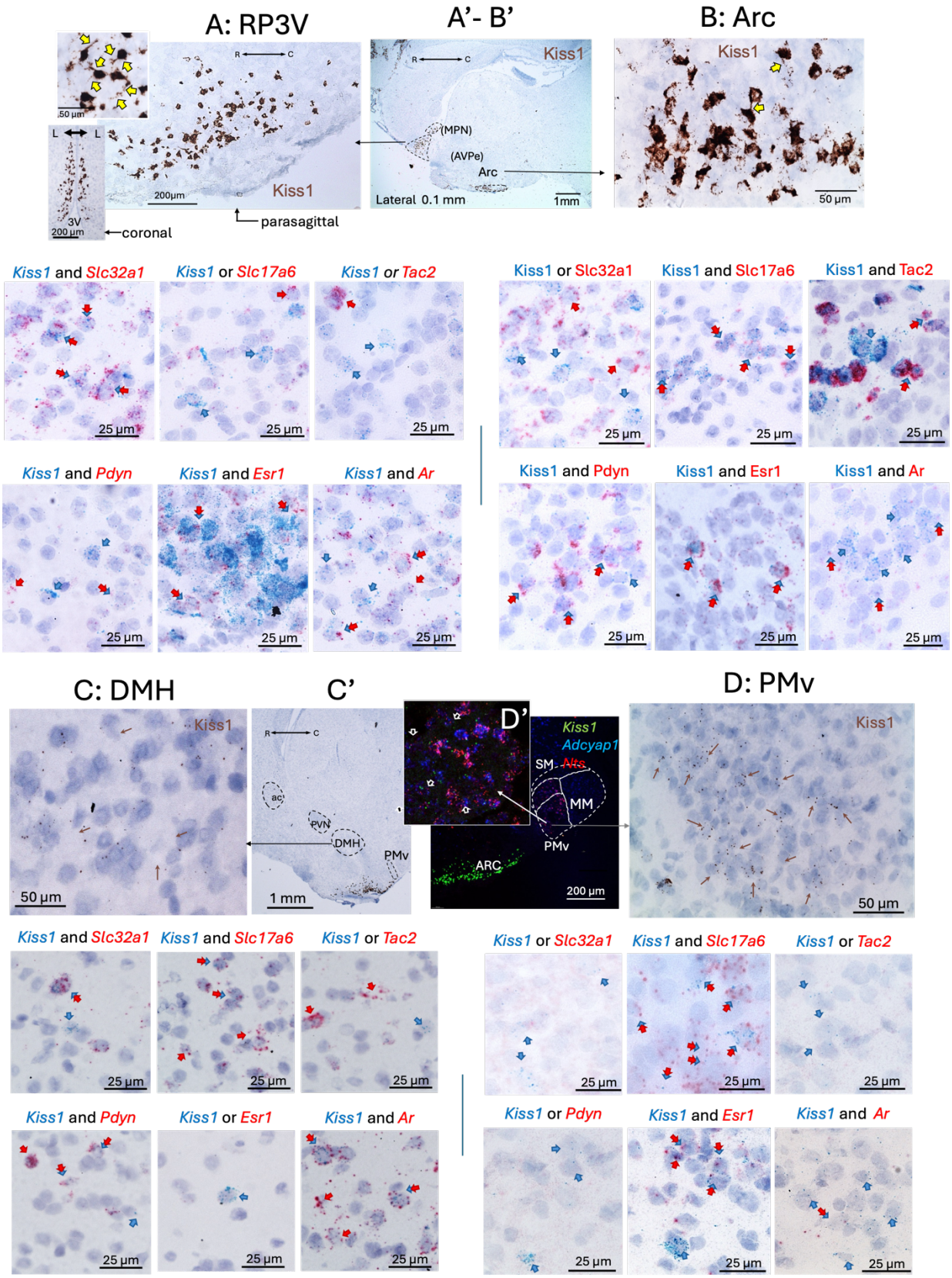
At the same rostrocaudal level as the $KP^{PMv}$ neurons is a small population of *Kiss1* expressing neurons in the medial postero-dorsal nucleus of the amygdala ($KP^{MeA}$, Fig 1E). After the Arc and RP3V KP populations, this extrahypothalamic population has been studied by several groups (Stephens and Kauffman 2017; Gresham et al. 2016; Pineda et al. 2017). Our DISH results (Fig. 1E, bottom panels) showed that the amygdalar *Kiss1* neurons co-expressed *Slc32a1 (mRNA for VGAT*) and a few co-expressed the mRNAs for VGLUT2 (*Slc17a6*). These neurons lacked co-expression of the mRNA for the peptides neurokinin (*Tac2*,) or dynorphin (*Pdyn*). In term of expression of sex steroids receptors, $KP^{MeA}$ neurons co-expressed *Ar* and at a lower level *Esr.* The chemoanatomical markers identified in this population of MePD cells confirmed by ISH at single cell level, a recent study in which the authors used RNA-seq to identify over 13,800 transcripts co-expressed by MeA neurons that were *Kiss1/ Cck /Cartpt* positive (Hatcher et al. 2023).

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

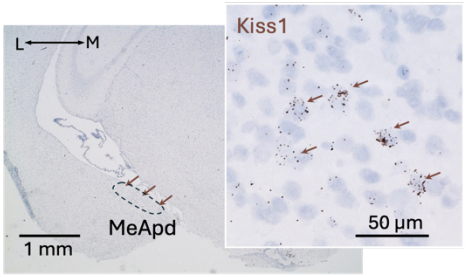
Dispersed neurons expressing *Kiss1* mRNA with similar molecular signatures as those of the medial amygdala group *($KP^{MeA}$)* were found surrounding the caudal part of the anterior commissure, spanning the septo-hypothalamic (SHy) area (Franklin and Paxinos 2008), and the stria terminalis (Fig. 1F), confirming previous reports using IHC (Xu et al. 2012), ISH (Di Giorgio et al. 2014; Stephens et al. 2018) and transgenic animals (Yamada et al. 2023). Here. we report that these neurons co-express *Slc32a1*, as well as *Esr1* and *Ar* (Fig. 1 F, bottom panels). No *Pdyn* or *Tac2* was observed in this neural population.

### 2.1.7 *Kiss1*-expressing neuronal populations in metencephalon

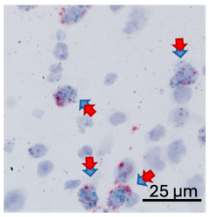
The metencephalic tractus solitarii (Sol) contains the most caudal population of KP neurons reported by kisspeptide immunoreactivity (Brailoiu et al. 2005; Dun et al. 2003). Here we show the expression of kisspeptin mRNA (Fig. 1G) in Sol. KP neurons express VGLUT2 (*Slc17a6*), approximately 40% of them express *Tac2*, 25% express *Esr1* and most of them express *Ar*. No coexpression of *Pdyn* or *Slc32a1* was observed (Fig. 2G).


A: RP3V
A'- B'
B: Arc
Kiss1
(MPN)
(AVPe)
Arc
Lateral 0.1 mm
1mm
50 µm
200µm
3V
200 µm
coronal
parasagittal
Kiss1 and Slc32a1
Kiss1 or Slc17a6
Kiss1 or Tac2
Kiss1 or Slc32a1
Kiss1 and Slc17a6
Kiss1 and Tac2
Kiss1 and Pdyn
Kiss1 and Esr1
Kiss1 and Ar
25 µm
C: DMH
C'
D'
D: PMv
Adcyap1
SM
MM
PMv
ARC
PVN
DMH
ac
1 mm
200 µm

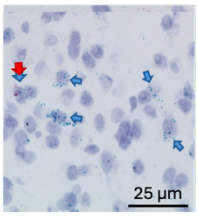
## E: MeA



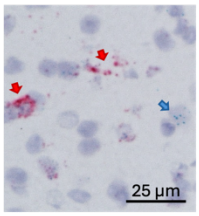

*Kiss1* and *Slc32a1*



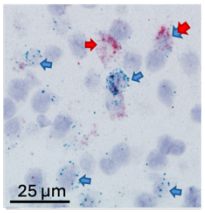

*Kiss1* and *Slc17a6*



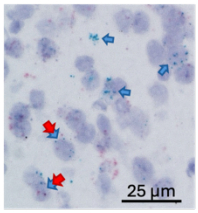

*Kiss1* or *Tac2*



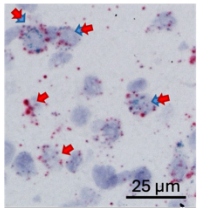

*Kiss1* and *Pdyn*



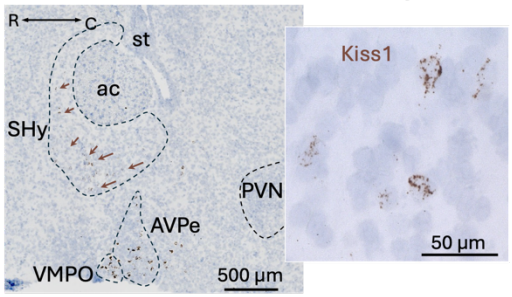

*Kiss1* or *Esr1*



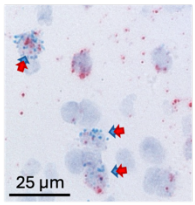

*Kiss1* and *Ar*



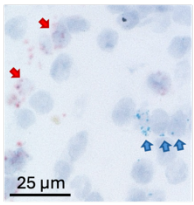

## F: SHy



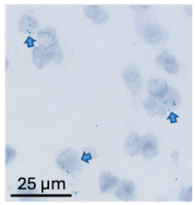

*Kiss1* and *Slc32a1*



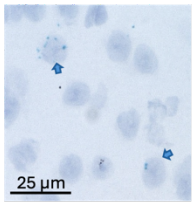

*Kiss1* or *Slc17a6*



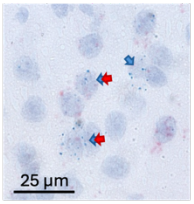

*Kiss1* or *Tac2*



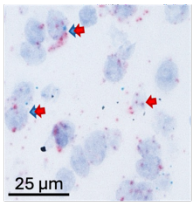

*Kiss1* or *Pdyn*



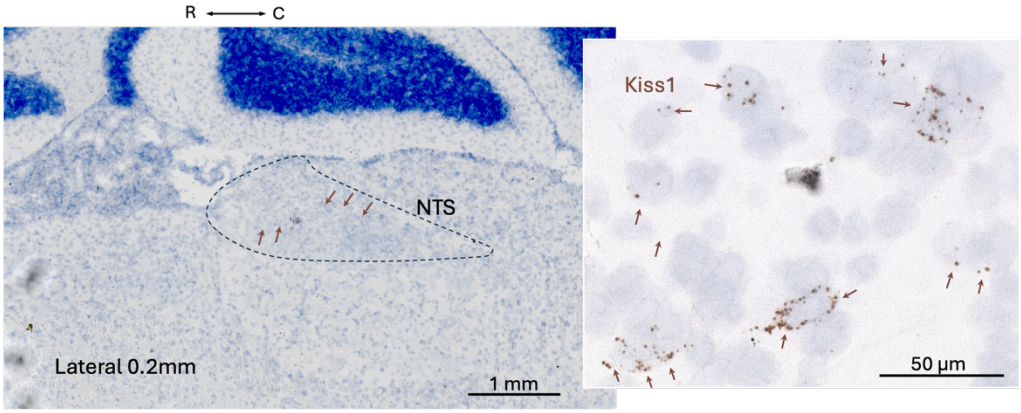

*Kiss1* and *Esr1*



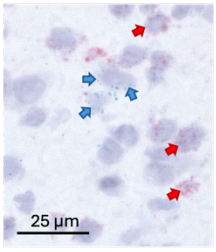

*Kiss1* and *Ar*



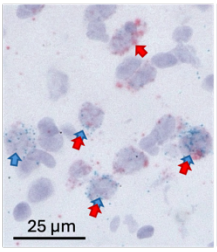

## G: NTS



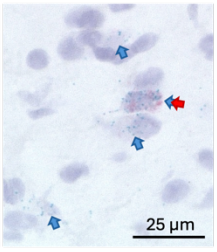

Kiss1 or Slc32a1



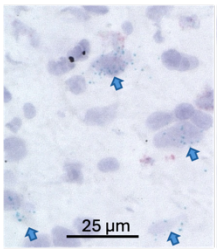

Kiss1 and Slc17a6



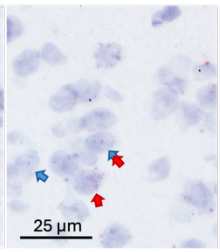

Kiss1 / Tac2




*Kiss1* or *Pdyn*




*Kiss1* or *Esr1*




*Kiss1* and *Ar*

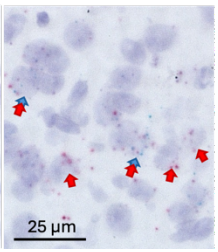


*Figure 1:* ***In situ hybridization (ISH) characterization of seven Kiss1 expressing neuronal populations (A-G) in the mouse brain.*** *Upper panels of each group show Kiss1 expression revealed by a highly sensitive singleplex reaction using RNAscope Brown kit method. Lower colored panels show mRNA coexpression using RNAscope duplex method which allowed us to observe the samples under light microscope with frequent magnification changes to precisely identify the neuroanatomical structures. A: the rostro-periventricular area of the hypothalamus ($KP^{RP3V}$) shown in a parasagital and in a coronal views in A´ and inset have a heterogeneal spatial distribution in the dorso-caudal axis and a steep decrease in density in the medial to lateral direction. The yellow arrow in the figures indicate cells with prominent expression of Kiss1 mRNA in the dendritic compartment. Lower 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 (Slc17a6), the neuropeptides neurokinin (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' and B : singleplex (brown) RNAscope showed Kiss1 epressing neurons in the arcuate nucleus some of them with dendritic Kiss1 expression (yellow arrows). Lower B-panels show that in this population Kiss1 is colocalized with Slc17a6, Tac2, Pdyn, Esr1 and Ar mRNAs but not with Slc32a. C and C': Kisspeptin expression in the dorso-medial hypothalamic area ($KP^{DMH}$). Singleplex RNAscope showed Kiss1 epressing neurons scattered in the DMH. The mRNA expression levels were low with approximately 5 puncta per cell in both sexes and gonadal conditions. Bottom panels show that in this population Kiss1 is colocalized with Slc17a6, Slc32a1and AR. No co-expression with Tac2, Pdyn, or Esr1 mRNAs was observed. D' and D Kisspeptin expression in the ventral premammillary nucleus area ($KP^{PMv}$). D': multiplex RNAscope fluorescent reaction shows that this newly characterized population of Kiss1 neurons co-express the mRNA for PACAP (Adcyap1) and neurotensin (Nts). D: High magnification photomicrograph of the PMv, taken from a section that was processed for Singleplex 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. Lower panels DISH photomicrographs show that this Kiss1 population co-express Slc17a6, Esr1 and AR mRNAs. E: Kisspeptin expression in the medial amygdala ($KP^{MeA}$)***.*** *Singleplex RNAscope showed Kiss1 expressing neurons concentrated in the posterodorsal part of the medial amygdala (MeApd). Duplex RNAscope 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 BNST complex ($KP^{SHy}$). scattered low Kiss1 expressing neurons were found around the anterior commisure in the septo-hypothalamic (SHy) area (brown arrows). The bottom panels show photomicrographs taken from the same area, 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 showed Kiss1 expressing neurons located in the solitary tract nucleus of male and female mice. The upper panels show representative photomicrographs from the area with neurons expressing Kiss1 at low abundance. Bottom 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.*

**2.2 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.

GNX reduced the dendritic expression of *Kiss1* in $KP^{RP3V}$. The *Kiss1* neurons of female proestrous mice were more strongly labelled, and a significanly 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 as the indicator (Fig. 2B) showed a significantly higher percentage of dendritic *Kiss1*-expressing neurons in male gonad intact mice (20.4 ±1.24%) compared to female proestrus mice (6.4 ±1.4 %). It is worth noting that in the proestrus of the estrous cycle, the plasma concentration of estradiol is at the highest level (Smith, Freeman, and Neill 1975). With respect to the sensitivity of these neurons to gonadectomy there was a potent upregulation of *Kiss1* dendritic expression 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 *Kiss1* mRNA puncta per cell in the $KP^{DMH}$, $KP^{PMv}$ and $KP^{NTS}$ showed no differences between male and female mice and as well no effects of gonadectomy in males or females (Fig. 2C, 2D, 2G).

We found that in $KP^{MeA}$ GNX induced a significant decrease in the average number of *Kiss1* mRNA puncta per cell only in male mice (Fig 2E) while Stephens and Kauffman (Stephens and Kauffman 2017) reported up-regulation of *Kiss1* in MeA after estrogen treatment of gonadectomized male mice, and it has been shown that aromatase expression is higher in male mice than in females (Stanic et al. 2014; Wu et al. 2009). We consider that it is likely that androgens exert direct effects on *Kiss1*-expression via signalling through the highly expressed *Ar* as well as indirectly through the *Esr1* after aromatization. Under this scenario it is likely that the sexually dimorphic response to gonadectomy in this population of neurons is because the drop in the local concentration of signalling trough the *Ar* iand *Esr1* is higher in males than in females.

Similarly to the $KP^{MeA}$ population, only male animals showed a decrease in the expression of *Kiss1* after gonadectomy, from 12.68 ± 1.0 to 7.62 ± 0.62 *Kiss1*-puncta per cell (Fig. 2 F). Interestingly, in these BST and in the MeA neurons it has been shown the existence of an inhibitory control on the expression of *Kiss1* via GABA-B receptors (Di Giorgio et al. 2014; Stephens et al. 2018), highlighting the plasticity of these neurons to other stimuli besides the sex steroids.

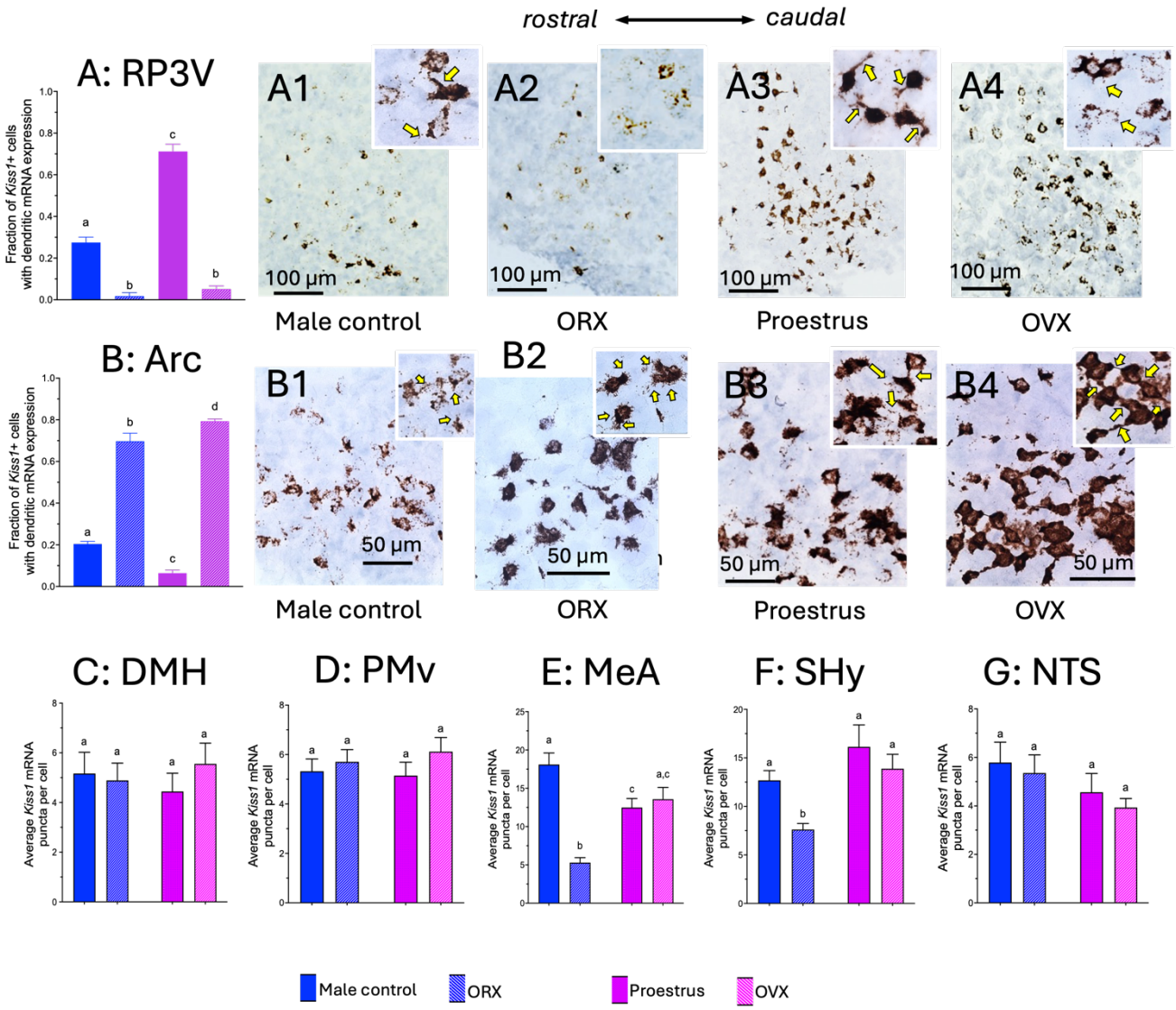



*Figure 2*: ***Effect of gonadectomy on Kiss1 expression in the seven Kiss1 expressing populations of the mouse brain.*** *A-B: The effects of gonadectomy were quantified in the two major population of Kiss1 expressing neurons, namely the RP3V (panels As) and Arc (panels Bs). The neurons in these regions 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.*

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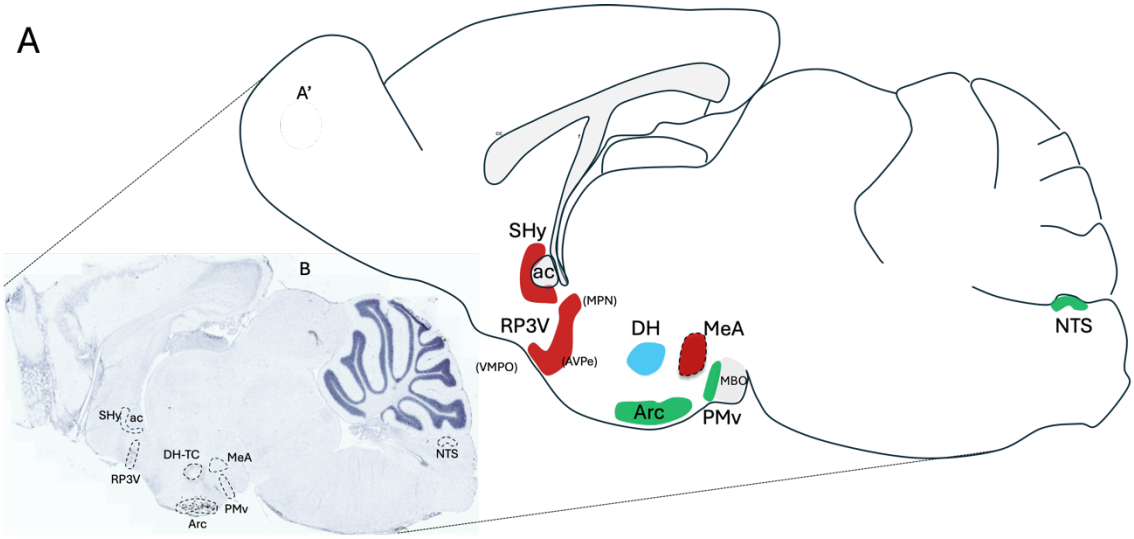
An overview of the seven population of neurons in the mouse brain, characterized with regard to the coexpression of KP mRNA (*Kiss1*) with *Slc32a1*, or *Slc17a6* is represented in the drawing of Fig 3A, in which the regions containing mainly *Kiss1* neurons co-expressing *Slc32a1* are symbolized in 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 (indicaded with a dotted line border), all the regions are located very close to the midline. The table in Fig 3B, summarize the molecular signatures of the seven population 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 ovarectomy (OVX) on *Kiss1* expression levels for each of the regions described in this study is presented in the table of 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 (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 commisure (ac); anteroventral periventricular nucleus (AVPe); arcuate nucleus (Arc); dorsomedial hypothalamic nucleus (DM); 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 the *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 (Bramham and Wells 2007) (Holt, Martin, and Schuman 2019; Kaneko et al. 2012; Liao et al. 2012).

For the $KP^{RP3V}$ population we showed 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 (Smith, Cunningham, et al. 2005; Smith, Dungan, et al. 2005; Kauffman 2010; Takumi et al. 2012), 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, the approach to quantify the percentage of *Kiss1* positive neurons that show dendritic expression avoids a possible selection bias originated by 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 (Lehman, Coolen, and Goodman 2010). 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 females (6.4%), and gonadectomy caused a sharp increase in dendritic mRNA labeling within *Kiss1*-expressing cells, to 70% in males and to 79% in females. The functional meaning of *Kiss1* dendritic expression in both $KP^{RV3V}$ 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.

It is worth noting that the majority of reports using in situ hybridization, are coincident in reporting a higher number of KP neurons in the RP3V of female rodents, however there is no clear agreement on sex differences of mRNA expression in the ARC, with some authors reporting no differences (Kauffman 2010; Di Giorgio et al. 2014), while others reporting a higher number of *Kiss1* expressing neurons in male animals (Smith, Cunningham, et al. 2005; Smith, Dungan, et al. 2005), and others reporting a higher number of *Kiss1*-expressing neurons in females (Takumi et al. 2012). These differences might be a consequence of the threshold that is set for determining cell *Kiss1*-positivity, the dense packing of neurons in the region, and the heterogeneous distribution along the rostro caudal or medio-lateral axis. The

quantification of the percentage of *Kiss1* neurons expressing dendritic mRNA might be a better measure for exploring this dynamic behavior.

A third group of KP neurons in the dorsomedial hypothalamus was identified and characterized here at the mRNA level. Previous studies using immunohistochemistry (Clarkson et al. 2009; Brailoiu et al. 2005; Clarkson and Herbison 2006) and Kiss1-CRE mice (Yeo et al. 2016; Cravo et al. 2011) 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 (Gottsch et al. 2004; Smith, Cunningham, et al. 2005). It has been suggested that this inconsistency between immunohistochemical and in situ hybridization histochemical findings might be explained by cross-reactivity between Kp and the peptide Arg-Phe-related peptide-1 (RFRP-1) (Oakley, Clifton, and Steiner 2009). Here we confirmed the existence of these dispersed neurons by RNAscope in situ hybridization, with a low somatic expression of Kiss1 mRNA in this region, and characterized the co-expression of Kiss1 with *Slc17a6* (Vglut2 mRNA), *Slc32a1* (VGAT mRNA) and *Ar* (androgen receptor mRNA) in this population. No significant changes were detected after gonadectomy.

In the ventral premammillary nucleus (PMv) *Kiss1* mRNA puncta were distributed homogeneously among the densely packed cells, which is clearly seen in parasagittal sections. The only report of the existence of this group of KP neurons was at the immunohistochemistry level in horse (Decourt et al. 2008) and in Kiss1-GFP trangenic mice (de Croft et al. 2012). 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 many other regions that never had been confirmed, raising concern about the possibility of supraphysiological ectopic expression of GFP. Here we confirmed by in-situ hybridization the existence of this population. These neurons do not share the molecular signatures of $KP^{Arc}$. Apart from expressing *Kiss1* and *Slc17a6,* the $KP^{PMv}$ neurons do not express *Tac2* nor *Pdyn,* express *Esr1* only weakly. Additionally, they 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 of AAV-aided tracing of genetically targeted PMv Adcyap1-Cre neurons yielded specific labeling in the hippocampal CA2 region, a brain region relevant for social behavior (Smith et al. 2016).

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 (Donato et al. 2013). The PMv is also important in linking leptin levels with changes in reproductive function (Donato et al. 2009). The classical model for this is that leptin induces an increased release of kisspeptin 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 activate the Arc Kisspeptin cells and these in turn activate the GnRH neurons (Petrine, Franci, and Del Bianco-Borges 2020). 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 (Elmquist et al. 1998). 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 (Yang et al. 2023).

The other three extrahypothalamic populations of KP neurons are located in the medial posterodorsal nucleus of the amygdala (MePD) in the septo-hypothalamic complex (SHy) and in the nucleus of the solitary tract (Sol). Besides the Arc and RP3V populations, the KP population in the amygdala has been studied most extensively (Pineda et al. 2017; Clarkson et al. 2009; Di Giorgio et al. 2014; Hatcher et al. 2023; Stephens et al. 2018; Xu et al. 2012). We confirmed the Kp-ir+ cell population is mainly confined within the *medial amygdala's postero-dorsal division* (MePD) which has been reported already elsewhere. These neurons co-expressed the vesicular GABA transporter VGAT (Slc32a1 mRNA) and a few number of Kiss1 neurons co-expressed the mRNAs for VGLUT2 (Slc17a6). In term of expression of sex steroids receptors, these *Kiss1*-expressing neurons co-expressed the mRNA for androgen receptor (Ar) and at a lower level the mRNA for estrogen receptor alpha *(Esr1).* The chemoanatomical markers identifyed in this population of MePD cells are congruent with a recent study in which the authors used RNA-seq to identify over 13,800 transcripts co-expressed by MeA neurons that were Kiss1/ Cck /Cartpt positive (Hatcher, Costanza et al. 2023). In this Kiss1 population, gonadectomy induced a decrease in the average number of *Kiss1* mRNA puncta per cell only in the male mice. The sixth KP population was widely distributed in the BNST complex, centered in the strio-hypothalamic nucleus ($KP^{SHy}$), with a spatial continuum spanning septo-strio-diagonal band of Broca ventrally. We refer to this group here as $KP^{SHy}$. This group showed a high similarity in molecular signature to the medial amygdala group ($KP^{MeA}$) that co-expresses *Slc32a1*, *Esr1*, and *Ar*. Similarly to MePD, in this population there was a decrease of the expression of KP mRNA puncta after gonadectomy only in males. The last population of extrahypothalamic KP neurons was found in the brain's major sensory hub in the dorsal medulla, the solitary tract nucleus ($KP^{NTS}$). The *Kiss1* neurons here co-expressed *Slc17a6, Tac2*, *Esr1* and *Ar.* Our results confirm by ISH the existence of this group of neurons reported previously by immunohistochemistry against metastin (Dun et al. 2003; Brailoiu et al. 2005). Additionally we characterized this population as glutamatergic (*Slc32a1*), and expressing *Tac2*, *Ar* and *Esr1*. KP neurons in NTS exhibited no effect of gonadectomy, in either males or females, on the average number of mRNA puncta per cell.

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 moleculr 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 (Lehman, Coolen, and Goodman 2010). 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 (Saldanha, Walters, and Fraley 2010). The third pattern of alteration by gonadectomy was down-regulation in males but no significant effect in female, in MEA and BST, 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. Technical consideration should be taking into account since we worked with thickness of 12 µm sections and selected one in every six sequential sections, making it feasible that we missed the narrow location where most of the KP neurons were situated. It is also possible that the modulation of other aspect of the chemotype of these neurons, such as glutamatergic or tachykinin traits, might be altered by GNX, a possibility not yet examined.

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 LHRH neurons. Kisspeptin fibers from these two major KP groups innervate other neurons besides the GNRH neurons, A recent study using electrophisiologcal recordings in combination with optogenetics, showed an excitatory input form the $KP^{Arc}$ and an inhibitory input from the $KP^{RP3V}$ converging onto preautonomic paraventricular (PVN) and dorsomedial (DMH) neurons to control their excitability (Stincic et al. 2021). How the post-synaptic effects are modulated by co-release of KP, glutamate and GABA from $KP^{RP3V}$ and $KP^{Arc}$ neurons is worthy of more detailed investigation.

There has been significant advance in our understanding of the critical role of kisspeptin in human sexually-related behavior in clinical studies featuring kisspeptin administration in men and women (Comninos et al. 2017). However, except for some scattered reports (e.g. (Pineda et al. 2017)), there has been little exploration of kisspeptin projections to areas other than LH-RH neurons of the hypothalamus. This is important because, as pointed out by Liu and Herbison (Liu and Herbison 2016), 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 required. 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 as it occurs endocrinologically, and individually, but also behaviorally, and socially.

## 2. Material and methods

## 2.1. Animals

C57BL/6 mice (N=40, male = 20, female = 20) 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 (Stout Steele and Bennett 2011). Briefly, 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 (Miller et al. 2012) to minimize incidence of testicular herniation. 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 Assessment of reproductive cycle

Vaginal smears were performed during consecutive days in control mice to collect brains of mice in proestrous stage (highest level of estradiol), 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 (Zhang et al. 2021; Zhang et al. 2020; Zhang et al. 2018) Briefly, 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.). After 7-8 weeks of gonadectomy, brains from control or gonadectomized mice were removed and rapidly frozen in pulverized dry ice. The fresh-frozen brains were sectioned in sagittal 12 µm thick sections with a Leica CM-1520 cryostat and mounted on positively charged Superfrost Plus microscope slides (Cat. 22-037-246, Fisher Scientific, Pittsburgh, PA). 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 (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. All experimental steps were performed following the provider's protocol. Briefly, after sectioning, slides were fixed in chilled 4% paraformaldehyde (Cat. 30525-89-4, Sigma-Aldrich) during 30 minutes, prepared in phosphate-buffered saline (Cat. M32631, Sigma-Aldrich), dehydrated in increasing concentrations of ethanol (50%, 70% and 100%) during 5 minutes each, delimited by a hydrophobic barrier, treated with hydrogen peroxide for 10 minutes, then treated with protease plus for 30 minutes at room temperature, incubated for two hours in RNAscope probes, incubated with the amplification-detection reagents following the provider's protocol, counterstained with hematoxylin and cover slipped.

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