Received: 17 September 2024 | Revised: 30 January 2025 | Accepted: 28 February 2025
DOI: 10.1111/jne.70019

ORIGINAL ARTICLE

Comprehensive chemoanatomical mapping, and the gonadal regulation, of seven kisspeptin neuronal populations in the mouse brain

Vito S. Hernández[1,2] | Mario A. Zetter[1,3] | Oscar R. Hernández-Pérez[1] | Rafael Hernández-González[4] | Ignacio Camacho-Arroyo[5] | Robert P. Millar[1,6,7] | Lee E. Eiden[2] | Limei Zhang[1,2]

[1]Department of Physiology, School of Medicine, National Autonomous University of Mexico (UNAM), Mexico City, Mexico
[2]Section on Molecular Neuroscience, NIMH-IRP, NIH, Bethesda, Maryland, USA
[3]Department of Medicine and Health, University of La Salle, Mexico City, Mexico
[4]Vivarium, School of Medicine, National Autonomous University of Mexico (UNAM), Mexico City, Mexico
[5]Research Unit in Human Reproduction, National Institute of Perinatology-Faculty of Chemistry, UNAM, Mexico City, Mexico
[6]Centre for Neuroendocrinology, University of Pretoria, Pretoria, South Africa
[7]Department of Integrative Biomedical Sciences, Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Cape Town, South Africa

Correspondence
Lee E. Eiden, Section on Molecular Neuroscience, NIMH-IRP, NIH, Bethesda, MD, USA.
Email: eidenl@nih.gov

Limei Zhang and Vito S. Hernández, Department of Physiology, School of Medicine, National Autonomous University of Mexico (UNAM), Mexico City, Mexico.
Email: limei@unam.mx and vitohdez@unam.mx

Funding information
PAPIIT-DGAPA-UNAM, Grant/Award Number: IG200121; National Institute of Mental Health, Grant/Award Number: MH002386; CONAHCYT, Grant/Award Number: CF-2023-G-243

Abstract

Kisspeptinergic signaling is well-established as crucial for the regulation of reproduction, but its potential broader role in brain function is less understood. This study investigates the distribution and chemotyping of kisspeptin-expressing neurons within the mouse brain. RNAscope single, dual, and multiplex in situ hybridization methods were used to assess kisspeptin mRNA (*Kiss1*) expression and its co-expression with other neuropeptides, excitatory and inhibitory neurotransmitter markers, and sex steroid receptors in wild-type intact and gonadectomized young adult mice. Seven distinct kisspeptin neuronal chemotypes were characterized, including two novel kisspeptin-expressing groups described for the first time, that is, the *Kiss1* population in the ventral premammillary nucleus and the nucleus of the solitary tract. *Kiss1* mRNA was also observed to localize in both somatic and dendritic compartments of hypothalamic neurons. High androgen receptor expression and changes in medial amygdala and septo-hypothalamic *Kiss1* expression following GDX in males, but not in females, suggest a role for androgen receptors in regulating kisspeptin signaling. This study provides a detailed chemoanatomical map of kisspeptin-expressing neurons, highlighting their potential functional diversity. 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 those of reproduction.

KEYWORDS
Ar, Esr1, gonadectomy, neuropeptides, vesicular amino-acid transporter

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
© 2025 The Author(s). *Journal of Neuroendocrinology* published by John Wiley & Sons Ltd on behalf of British Society for Neuroendocrinology.

1 | INTRODUCTION

The kisspeptins, which include the precursor KP-54 and the proteolytically processed 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 (deorphanized from GPR54, KiSS1R),[4] are important for cell signaling within the hypothalamus in regulating reproductive function through stimulation of gonadotropin-releasing hormone (GnRH) secretion.[5–14] KP neurons are also located within extrahypothalamic circuits, notably in the amygdala, where emotion and cognition are integrated and putatively 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]; the most prominent being in the arcuate nucleus (Arc) and in the rostroperiventricular region of the third ventricle (RP3V). This region includes the periventricular nucleus (PeN), the anteroventral periventricular nucleus (AVPV), and some scattered cells in the anterodorsal preoptic area (ADP). The other regions that have been reported to express *Kiss1*, albeit at lower levels, are the medial amygdala, the bed nucleus of the stria terminalis—the lateral septal nuclei, 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 nutrient state.[27–33] Gonadal status and kisspeptin signaling exert mutual complex regulation of the hypothalamic–pituitary–gonadal (HPG) axis.

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 dubbed "KNDy" neurons for this reason)[34–36] as well as galanin.[37] Neurokinin B and dynorphin seem to act autosynaptically on kisspeptin neurons in the Arc to synchronize and regulate the pulsatile secretion of kisspeptin and the 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.[38,39] TRPC channels are a subfamily of nonselective cation channels that are part of the TRP superfamily.[40,41] They are calcium-permeable and receptor-operated, and are activated by the phospholipase C (PLC) signaling pathway.[39] 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.[42,43] KP direct excitatory effects on GnRH neurons can be potently modulated by GABA, via $GABA_A$ and $GABA_B$ receptors, and by glutamate, in a spatial–temporal and steroid-dependent manner.[12–14,44] Kisspeptins can also activate non-GnRH neurons that synapse on GnRH neurons, exerting indirect effects on GnRH neuronal responses by modulating fast synaptic transmission through GABA and glutamate.[45,46] 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, as well as non-HPG neuronal activity controlling various behaviors.

In pursuing an understanding of the roles of KP in these non-hypothalamic brain regions, detailed chemotyping of KP neurons throughout the brain must be undertaken at a high level of resolution.

The objectives of our study were, first, to characterize kisspeptinergic neuronal populations based on their molecular signatures and distribution throughout the wild-type, intact adult mouse brain using single, dual, and multiplex RNAscope methods, and secondly, to assess the impact of short-term 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, including two novel kisspeptin-expressing groups described for the first time, that is, the *Kiss1* populations in the ventral premammillary nucleus and in the nucleus of the solitary tract. *Kiss1* mRNA was also observed in both somatic and dendritic compartments of hypothalamic neurons. High androgen receptor expression and decreased *Kiss1* expression in medial amygdalar and septo-hypothalamic regions following GDX in males, but not in females, suggest a role for androgen receptors in regulating kisspeptin signaling. 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 young adult mice of 25–30 g (10–12 weeks old) were obtained from the local animal vivarium, distributed in four groups ($N = 24$, intact males [$n = 8$] and females [$n = 8$] and gonadectomized male [$n = 4$] and female [$n = 4$]). Mice were housed in three per cage under controlled temperature and illumination (12 h/12 h) 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 LMCR-08).

2.2 | Assessment of reproductive cycle and gonadectomy (GNX)

Vaginal smears were conducted on consecutive days in control female mice in order to collect brains at the proestrus stage.[47,48] Briefly, the mice were gently restrained by hand, and the vulva was exposed. A

pipette with a 100 μm tip was used to wash the vaginal cavity with 10 μL of isotonic saline. The liquid was then deposited on a clean glass slide and left to dry. Slides were counterstained with crystal violet stain (0.1%) and observed under a light microscope using 10× and 20× objectives. Proestrus is characterized by the absence of leucocytes and the presence of numerous oval nucleated epithelial cells.

The detailed procedures of mouse ovariectomy (OVX) and orchiectomy (ORX) were described elsewhere.[49] Briefly, a dorsal approach was used to remove the ovaries under ketamine (75 mg/kg) and xylazine (16 mg/kg) anesthesia. After preparing the surgical site, a 1 cm dorsal incision was made, and the abdominal wall was cut laterally to access the ovaries. Ovarian vessels and ligaments were ligated and cut, and the muscle and skin incisions were closed with sutures and surgical glue. Post-surgery, animals were monitored for infection and swelling, and sutures were removed within 14 days. Ketoprofen (5 mg/kg) was given subcutaneously daily for three days.

An abdominal approach was used to remove the testicles, under the same anesthesia protocol, to minimize herniation risks. A 1 cm incision was made through the skin and abdominal wall, and the testicular fat pad was followed to locate and excise the testicle after ligating the vas deferens and blood vessels. The incision was closed in layers using absorbable sutures for the muscle and non-absorbable sutures with surgical glue for the skin.

Gonadectomy success was confirmed by the observation of physical removal of the gonads bilaterally, post-mortem, in males and also by performing vaginal smears during consecutive days in females. In the ovariectomized mice, only abundant leukocytes and some cornified epithelial cells were observed.

2.3 | RNAscope single, dual, and multiplex in situ hybridization (SISH, DISH, MISH) procedures

Detailed methods are described elsewhere.[50–52] Briefly, intact and GDX mice (after 7–8 weeks of gonadectomy) were deeply anaesthetized with sodium pentobarbital (100 mg/kg b.w., i.p.) and decapitated using a small animal guillotine. Brains were removed and rapidly frozen in pulverized Dry ice. The fresh-frozen brains were sectioned in sagittal and coronal planes, of 12 μm thickness, with a Leica CM-1520 cryostat and mounted on positively charged microscope slides. Every fourth successfully mounted serial section was processed for *Kiss1*-single ISH (SISH) reaction using the Brown-kit method. SISH experimental procedures were performed according to the manufacturer's instructions (*Singleplex*, ACDBio.com).

To evaluate the co-expression of relevant and available mRNAs in the seven populations identified with the SISH experiment, we performed DISH and MISH experiments according to manufacturer instructions (ACDBio.com). The following six probes of channel 2 were used for the DISH reaction, combined with the kisspeptin channel 1 probe (Mm-*Kiss1*, Cat No. 500141): VGAT (Mm-*Slc32a1*-C2, Cat No. 319191-C2), VGLUT2 (Mm-*Slc17a6*-C2, Cat No. 428871-C2); neurokinin B (Mm-*Tac2*-C2, Cat No. 446391-C2), dynorphin (Mm-*Pdyn*-C2, Cat No. 318771-C2), estrogen receptor alpha (Mm-*Esr1*-C2, Cat No. 496221-C2), and androgen receptor (Mm-*Ar*-C2, Cat No. 316991-C2). For the MISH reaction, the following channel 2 and 3 probes were added to the designed reaction: PACAP (Mm-*Adcyap1*-*C2*, Cat No. 405911-C2) and neurotensin (Mm-*Nts*-C3, Cat No. 420441-C3).

Six adjacent 12 μm-thick sections were mounted on the same slide. Each section on a given slide was reacted with one of the above combinations. This measure was designed to minimize the possible spatial variations, due to the heterogeneous nature of *Kiss1*-expressing cell distribution, that could affect the co-expression percentage calculation among the probe combinations. In order to precisely localize the anatomical structures, we mounted a seventh section on a reference slide for Nissl staining before continuing mounting on the subsequent slide for DISH reaction.

The experimental design for sample collection and distribution on slides, along with probe combinations for single and double in situ hybridization experiments (SISH, A and DISH, B) is shown in detail in Figure S1. The notations beneath each panel explain the advantages and limitations of these designs, particularly highlighting why statistical methods of extrapolation are inapplicable for this study.

2.4 | Microscopical examination, imaging, and analysis

Sagittal and coronal quasi-serial sections (1 in 4 for SISH and 1 in 7 for DISH, see Figure S1) were first examined using a Nikon Eclipse E600 microscope, along with its digital camera for photographic documentation. Low-magnification examination was conducted to localize anatomical structures, referencing Paxinos and Franklin's The Mouse Brain in Stereotaxic Coordinates,[53] in conjunction with the Allen Mouse Brain Atlas (https://mouse.brain-map.org/, Allen Institute for Brain Science), specifically consulting the *Slc32a1*, *Slc17a6*, *Tac2*, *Pdyn*, *Esr1*, and *Ar* gene expression data.

Once a *Kiss1*-expressing brain structure was identified, we counted the cells co-expressing channel-1 (*Kiss1*) and one of the six channel-2 probes, relative to the total channel-1-expressing cells in Nissl-labeled nuclei within on-screen micrographs taken at 40× magnification (Nikon Eclipse E600 microscope), within an identified anatomical structure. Each micrograph is considered as a "region of interest" (ROI). The micrograph covers 0.06 mm^2 (292 μm × 212 μm). At least one set of micrographs (one for each of the six mRNA-probe combinations) was obtained for each anatomical region/subject (i.e., $n \geq 8$ ROIs per each mRNA-probe combination). The average co-expression ratios for a given combination from $n \geq 8$ ROIs per each anatomical structure/subject are expressed in percentile intervals (i.e. "+": 1%–25%, "++": 26%–50%; "+++" 51%–75%; "++++": 76%–100%). Generally, there were at least 3 *Kiss1* cells per ROI in the regions where the *Kiss1* cell densities are low, such as the NTS, MEA, SHy, and DMH. We used this semi-quantitative expression instead of precise numeric values due to the high variability of *Kiss1* expressing cell density encountered among sections/subjects, from four of the seven anatomical structures (vide infra and also consult the Figure S1

for detailed sample selection and probes-cocktail distribution). We consider that this expression allows us to confidently express the central tendencies of the observed biological phenomena without ignoring the natural data dispersion reflecting individual/sampling differences.

To assess the GNX effect on *Kiss1* expression, we evaluated two parameters. For the two *Kiss1*-expressing neuronal populations in the hypothalamus with observed dendritic *Kiss1* labeling (i.e., Arc and RP3V regions), we used the ratio of cells with dendritic *Kiss1* expression to total *Kiss1*-labeled cells in a 0.04 mm^2 area (projected on a computer monitor under a 40× objective of a Nikon Eclipse E600 microscope). This was averaged over two areas for each of the six animals per group. For other regions with significantly lower *Kiss1* mRNA expression, we identified *Kiss1* labeling over Nissl-stained nuclei and averaged the puncta for all identified cells per experimental condition (see Section 3 for a detailed discussion).

A single RNA punctum was considered a positive cell (although this single-punctum occurrence is quite rare), as these RNAscope methods (SISH, DISH, and MISH) generally show high sensitivity and low background. Additionally, we do not have another objective criterion based on pre-existing knowledge.

2.5 | Statistical analysis

Statistical analysis was performed using GraphPad Prism 10 Software. Data were assessed for normality using the 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/Nissl-stained nuclei between groups for each of these regions, a two-way ANOVA with sex and treatment as factors was used. A Tukey post hoc test was used for pairwise comparisons. 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 | Kisspeptin neuronal group in the rostro-periventricular area of the hypothalamus (KP^{RP3V})

The RP3V region (Figure 1A) hosts one of the two major Kiss1-expressing neuronal groups in wild-type intact mice, which have been extensively studied over the past two decades. This area encompasses *Kiss1* neurons distributed throughout the ventromedial preoptic nucleus (VMPO), anteroventral periventricular nucleus (AVPV), periventricular nucleus (Pe), anterodorsal preoptic area (ADP), and median preoptic nucleus (MnPO) (Figures 1A and S1). The density of *Kiss1*-expressing neurons is heterogeneous. In a parasagittal view (Figure 1A1), heterogeneously distributed Kiss1-labeled cells can be observed around lateral 0.05 mm, extending approximately 300 μm rostro-caudally and 800 μm ventrodorsally, with a decreasing density gradient upwards. In the median preoptic nucleus, sparsely distributed *Kiss1*-expressing neurons are found (Figure 1A1a). In contrast, in a coronal view (Figure 1A2, bregma 0.02 mm), *Kiss1*-labeled cells are located in relatively narrow bands alongside the wall of the third ventricle, with most cells clustered within a <100 μm band on each side, and density decreasing sharply in the mediolateral dimension (Figure 1A2a).

Kiss1 is extensively expressed in proximal dendrites of the KP^{RP3V} population, in addition to cell bodies (Figure 1 inset, yellow arrows). This phenomenon has been interpreted to suggest local dendritic translation of mRNA. Dendritic *Kiss1* expression was observed in both male and female brains.

The RNAscope DISH method allows evaluation of the co-expression of separate mRNAs within single cells (Figure 1A3). This *Kiss1* neuronal population co-expresses *Slc32a1* (mRNA for VGAT, Figure 1A3a) and some co-express *Slc17a6* (mRNA for VGLUT2, Figure 1A3b). Some *Kiss1* neurons express *Pdyn* (mRNA for dynorphin Figure 1A3c) but are negative for *Tac2* (mRNA for neurokinin B, Figure 1A3d). The KP^{RP3V} group strongly co-expressed *Esr1* and *Ar* (mRNAs encoding the estrogen receptor alpha and androgen receptor respectively, Figure 1A3e,f, 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 ERa and AR.

3.2 | 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 (Figure 1B, a sagittal view of KP^{Arc} at lateral 0.20 mm). These cells also showed both somatic and dendritic expression of *Kiss1* (Figure 1B1a,b, yellow arrows). The cells are densely distributed toward the ventral floor of the arcuate nucleus with a rostro-caudal span of around 1 mm (Figure 1B1a). The density decreases dorsally (Figure 1B1a). In Figure 1B1a,B2a, the *Kiss1* population is shown from a coronal view at bregma −1.70 mm, in a spherical shape with a diameter of approximately 250 μm on each side of the third ventricle. Using DISH (Figure 1B3), we confirmed the co-expression of mRNAs for the vesicular glutamate transporter 2 (*Slc17a6*, Figure 1B3b), neuropeptides neurokinin B (*Tac2*, Figure 1B3d) and dynorphin (*Pdyn*, Figure 1B3c), as previously reported.[34,36,54] *Kiss1*-positive neurons expressed both estrogen receptor alpha (*Esr1*, Figure 1B3e) and androgen receptor (*Ar*, Figure 1B3f) mRNAs. KP^{Arc} neurons did not co-express *Slc32a1*, the mRNA encoding VGAT (Figure 1B3a).

3.3 | Kisspeptin neuronal group in the dorsomedial hypothalamic area (KP^{DMH})

Kiss1-expressing neurons were found in the dorsomedial hypothalamic region (DMH, Figure 1C), beginning immediately caudal to the

JNE Journal of Neuroendocrinology —WILEY

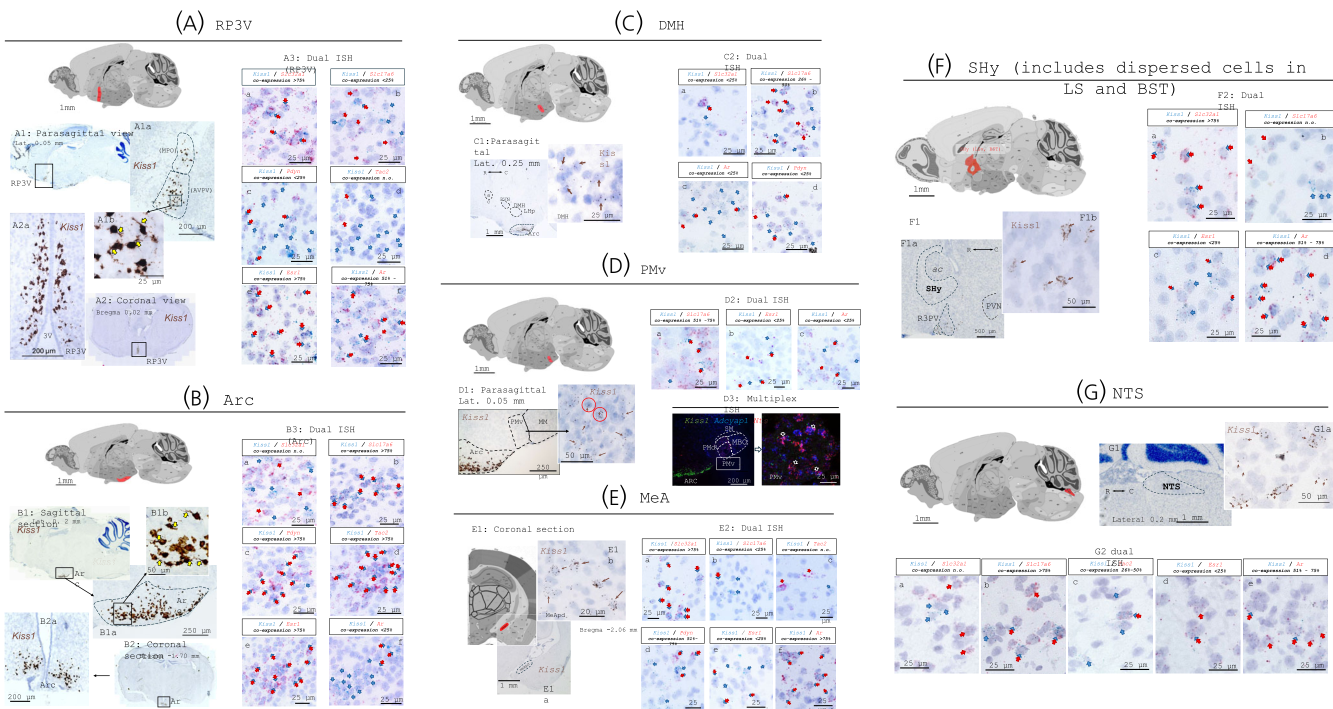


FIGURE 1 Anatomical localization and chemotyping of seven *Kiss1*-expressing neuronal populations in the intact mouse brain (A and B: From female mice in proestrus and C-G from male mice). (A) KP population in the rostro-periventricular area of the hypothalamus (KP^{RP3V}) shown in a parasagital (A1 and inset) and in a coronal view (A2) 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 *Slc32a1*, *Slc17a6*, *Tac2*, *Pdyn*, *Esr1 or Ar*. Double arrows show that in this region *Kiss1* is co-expressed with *Slc32a1*, *Pdyn*, *Esr1* and *Ar*. The average ratio of co-expression for anatomical structure per probe combination is expressed in quartile intervals for each micrograph. (B) Kisspeptin population in the arcuate nucleus of the hypothalamus (KP^{ARC}). B1b and inset show *Kiss1* expressing neurons with dendritic *Kiss1* expression (yellow arrows). B_3 parasagittal micrographs show that in this population *Kiss1* is colocalized with *Slc17a6*, *Tac2*, *Pdyn*, *Esr1*, and *Ar* mRNAs but not with *Slc32a1*. (C) *Kiss1* population in the dorsomedial hypothalamic area (KP^{DMH}). *Kiss1* detected by singleplex RNAscope is shown in a parasagittal section in C1, *Kiss1*-expressing neurons in the DMH are indicated by brown arrows in the inset. Duplex RNAscope micrographs in C2 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 expressing neuronal population in the ventral premammillary nucleus (KP^{PMv}). D1 and inset: Micrograph of the PMv, taken from a parasagittal section shows *Kiss1* expressing neurons scattered in the PMv, the expression levels of *Kiss1* were low. DISH (D2) and MISH (D3) RNAscope reactions show that this newly characterized population of *Kiss1* neurons co-express the mRNAs for *Slc17a6* (D2a), *Esr1* (D2b), *AR* (D2c), PACAP and neurotensin (*Adcyap1* and *Nts* respectively, D3). (E) *Kiss1* expressing population in the medial amygdala (KP^{MeA}). Panel E_1, low and high magnification micrographs of a coronal section showing *Kiss1* expressing neurons concentrated in the posterodorsal part of the medial amygdala (MeApd). E2, Duplex RNAscope showed that *Kiss1* neurons strongly co-expressed *Slc32a1*(E2a) and *Ar* (E2f) and to a lower level also expressed *Slc17a6* (E2b), *Esr1* (E2e), and *Pdyn* (E2d). (F) *Kiss1* expressing population in the septo-hypothalamic area (KP^{SHy}). Panel F1, parasagittal section depicts the SHy area, spanning septal, BNST, and hypothalamic regions where scattered low *Kiss1*-expressing neurons (inset of F1) were found around the anterior commissure. F2, micrographs taken from SHy in slices treated for RNAscope Duplex. *Kiss1* was observed to be colocalized with *Slc32a1* (F2a), *Esr1* (F2c), and *Ar* (F2d). No co-expression with *Tac2*, *Pdyn*, or *Slc17a6* (F2b) mRNAs was observed in this region. (G) *Kiss1* expressing population in the NTS (KP^{NTS}). Singleplex RNAscope (G1), showed *Kiss1* expressing neurons located in the solitary tract nucleus. G1 and G1a, 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* (G2b), *Tac2* (G2c), *Esr1* (G2d), and *Ar* (G2e), no co-expression with *Slc32a1* (G2a) or *Pdyn* was observed.

paraventricular nucleus posterior division and extending to the lateral posterior hypothalamic region (LHp, Figure 1C1). The cells of this group had low *Kiss1* expression (Figure 1C1, inset). Analysis of DISH experiments (Figure 1C2) showed that about half of the KP^{DMH} cells express *Slc17a6* (Figure 1C2b), and some express *Slc32a1* (Figure 1C2a), *Ar* (Figure 1C2c) and *Pdyn* (Figure 1C2d), but not *Esr1* or *Tac2* mRNAs (micrographs not shown).

3.4 | Kisspeptin neuronal group in the ventral premammillary 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, Figure 1D). These neurons had a low expression of KP (average 5–6 puncta per cell) but were densely packed and

homogeneously distributed throughout the PMv (Figure 1D1, inset). Using dual and multiplex in situ hybridization experiments (Figure 1D2), we showed that these *Kiss1*+ neurons co-express the vesicular glutamate transporter 2 (*Slc17a6*, Figure 1D2a) mRNA and show a low expression of *Ar* and *Esr1* (Figure 1D2b,c). They also express the mRNA for PACAP (*Adcyap1*) and neurotensin (*Nts*) (Figure 1D3). No co-expression with *Tac2* or *Pdyn*, mRNAs that are strongly expressed in KP neurons of the adjacent arcuate region, was observed.

3.5 | *Kiss1*-expressing neuronal populations in the medial amygdala (KP^{MeA})

A population of *Kiss1*-expressing neurons exists in the medial postero-dorsal nucleus of the amygdala (KP^{MeA}, Figure 1E,E1a,b). This extrahypothalamic population has been studied by several groups.[17,19,55] The amygdalar *Kiss1* neurons co-expressed *Slc32a1* (mRNA for VGAT, Figure 1E2a), with a few expressing VGLUT2 (*Slc17a6*, Figure 1E2b). Some of these neurons co-express dynorphin (*Pdyn*, Figure 1E2d), but not neurokinin B (*Tac2*, Figure 1E2c). KP^{MeA} neurons co-expressed *Ar* and, at a lower level, *Esr1* (Figure 1E2e,f).

3.6 | *Kiss1*-expressing neuronal populations in the septo-hypothalamic nucleus (KP^{SHy}) and surrounding area of the anterior commissure extending to the lateral septum (LS) and bed nucleus of the stria terminalis (BST)

Sparsely distributed *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 ventral lateral septum-strio-hypothalamic subfields,[56] (Figures 1F,F1a, b and 2, panels As, green arrows for a parasagittal view and Figure S2 panel A and A1, bregma 0.38 mm and panel B and B4, bregma 0.02 mm for a coronal view of this population). Note that at the coronal plane 0.02 mm, this population of cells can be seen within a region delineated by three brain conducting systems, that is, fornix (f), stria terminalis (st), anterior commissure (ac), confirming previous reports using IHC,[49,57] ISH, and transgenic-reporter animals.[24,33,58–60] Considerations regarding a discrepancy in the *Kiss1* population in the lateral septal nucleus—between our observations and previous reports using various genetic or pharmacological/hormonal manipulations—are presented in Section 4.1, with a supporting figure in Figure S1.

These neurons co-express *Slc32a1* (Figure 1F2a), as well as *Esr1* and *Ar* (Figure 1F2c,d). No *Slc17a6* (Figure 1F2b), *Pdyn*, or *Tac2* were detected in these neurons (micrograph not shown).

3.7 | *Kiss1*-expressing neuronal populations in the nucleus of the tractus solitarii (NTS)

The metencephalic nucleus tractus solitarii (NTS) contains the most caudal population of KP neurons. This group of neurons was reported using metastin immunoreactivity in rat.[61,62] Here, we show the expression of kisspeptin mRNA (Figure 1G1,G1a) in the NTS of mouse. Analysis of DISH experiments (Figure 1G2) showed that KP neurons expressed VGLUT2 (*Slc17a6*, Figure 1G2b), with approximately 40% of them expressing *Tac2* (Figure 1G2c), 25% *Esr1* (Figure 1G2d), and most expressing *Ar* (Figure 1G2e). No co-expression of *Pdyn* or *Slc32a1* (Figure 1G2a) was observed. 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.

3.8 | *Kiss1* expression's vulnerability in 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* puncta, cannot objectively reflect the observed reduction/augmentation. We therefore 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 labeled, and a significantly higher ($p < 0.001$) percentage of them showed dendritic mRNA expression, compared with males (71.1% ± 3.0% in females vs. 27.6% ± 2.2% in males); see Figure 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% ± 0.5%, $p < 0.001$ 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 (Figure 2B) showed a significantly higher ($p < 0.001$) percentage of dendritic *Kiss1*-expressing neurons in intact male mice (20.3% ± 1.1%) compared with female proestrus mice (6.4% ± 1.4%). After gonadectomy, there was a significant ($p < 0.001$) upregulation of *Kiss1* dendritic expression in the Arc region in both sexes, with the expression levels in females surpassing those of males (ORX: 69.8% ± 3.42% vs. OVX: 79.3% ± 1.1%, $p < 0.01$).

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 (Figure 2C,D,G).

In KP^{MeA}, GNX induced a significant decrease in the average number of *Kiss1* mRNA puncta per cell only in male mice (Figure 2E, intact: 18.1 ± 1.5 vs. ORX: 5.3 ± 0.6, $p < 0.001$). 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 (Figure 2F).

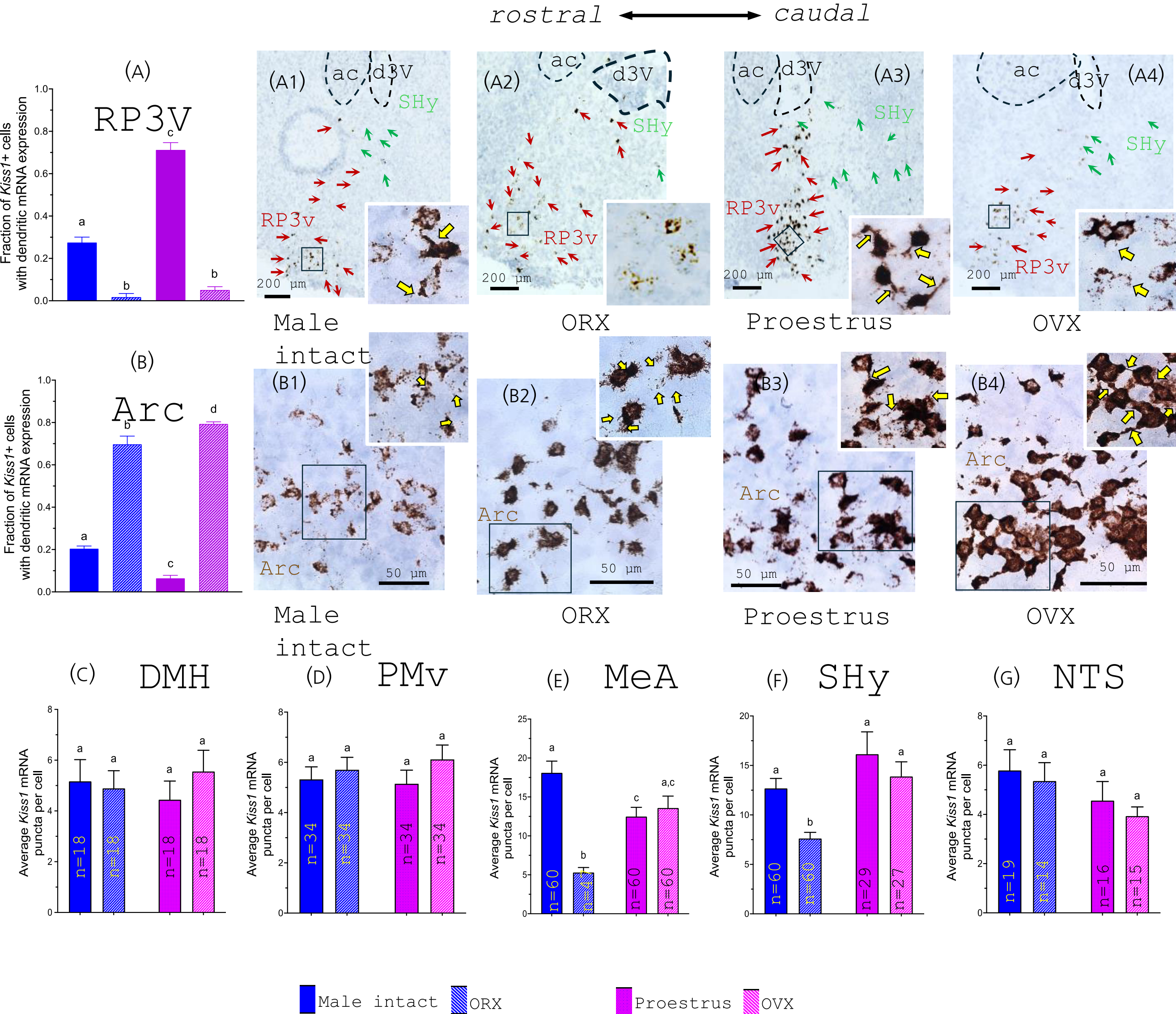
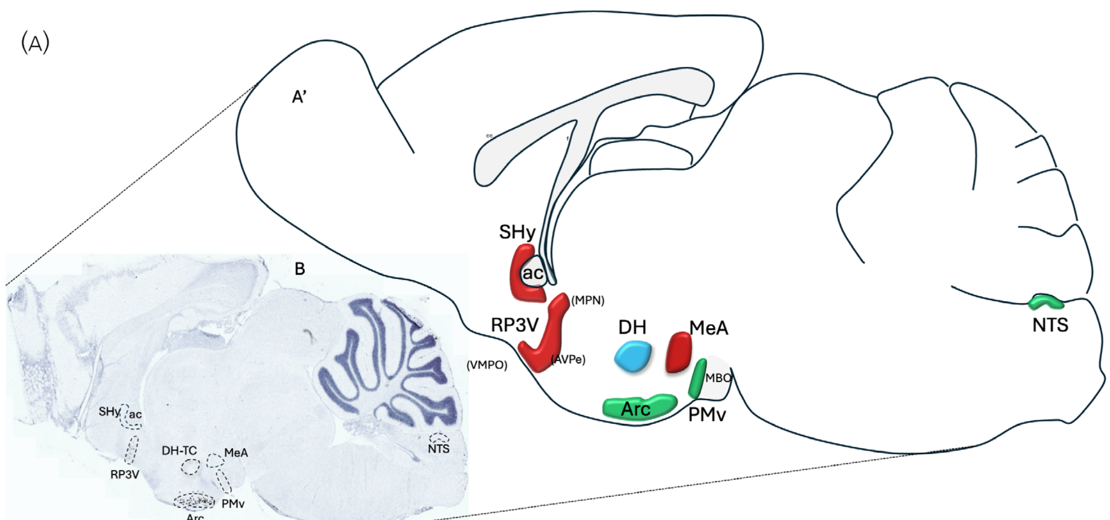


FIGURE 2 Effect of gonadectomy on *Kiss1* expression in the seven Kiss1 expressing populations of the mouse brain. (A, B) The effects of gonadectomy were quantified in the two major population of Kiss1 expressing neurons, namely the RP3V (panels As) and Arc (panels Bs). The neurons in these regions showed 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. For these two regions, the bar graphs represent the average of twelve areas were the fraction of dendritic *Kiss1* expression was evaluated in photomicrographs taken from matched regions of interest in each condition. Representative photomicrographs of RP3V (A1–A4) and Arc (B1–B4) show low and high magnification of these areas in male (A1 and B1) and female proestrus (A3 and B3) control mice and after orchidectomy (ORX, A2 and B2) and ovariectomy (OVX, A4 and B4). Yellow arrows indicate examples of neurons with dendritic expression. In the RP3V the fraction of cells expressing dendritic *Kiss1* is higher in proestrus 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. For these regions we quantified the average mRNA puncta per cell. The number of cells used to obtain these results is indicated within each bar. 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 Figure 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. Figure 3B summarizes the molecular signatures of the seven populations of kisspeptinergic neurons, indicating the co-localization of *Kiss1* with *Slc32a1*, *Slc17a6*, *Pdyn*, *Tac2*, *Esr1*, and *Ar*. A semiquantitative report on the co-expression ratio of these mRNAs with *Kiss1* and a summary of the effect of orchidectomy (ORX) and ovariectomy (OVX) on *Kiss1* levels for each of the regions described in this study are presented in Figure 3B.

4 | DISCUSSION

We report here the neuroanatomical mapping and the molecular signatures of seven distinct *Kiss1*-expressing populations and their



(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.	++	+	+++	↓	=
SHy	++++	n.o.	n.o.	n.o.	+	+++	↓	=
DMH-LH	+	++	n.o.	+	n.o.	+	=	=
Arc	n.o.	++++	++++	++++	++++	+	↑	↑
PMv	n.o.	+++	n.o.	n.o.	+	+	=	=
NTS	n.o.	++++	++	n.o.	+	+++	=	=

FIGURE 3 Molecular signature of seven population of *Kiss1* neurons in the brain. (A) Parasagittal section and representative drawing of the regions observed to host *Kiss1* positive neurons. The regions that have mainly GABAergic (*Slc32a1*) neurons are colored in red, the regions with mainly glutamatergic neurons (*Slc17a7*) are colored in green, and the DM region colored in blue, contains glutamatergic and GABAergic Kiss1 neurons. (B) Semiquantitative report of ratios between *Kiss1* cells co-expression one of the six mRNAs vs. total *Kiss1* expressing cells in a given ROI (see Section 2.4), in percentile intervals: "+": 1%–25%, "++": 26%–50%; "+++" 51%–75%; "++++": 76%–100%). 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*). AC, anterior commissure; Arc, arcuate nucleus; AVPe, anteroventral periventricular nucleus; DMH, dorsomedial hypothalamic nucleus; MBO, mammillary bodies; MeA, medial amygdala; MPO, median preoptic nucleus; NTS, nucleus of tractus solitarius; PMV, ventral premammillary nucleus; RP3V, rostro-periventricular region of the third ventricle; SHy, septo-hypothalamic area; VMPO, ventromedial preoptic area.

sensitivity to gonadectomy. While others have independently reported that kisspeptin is co-expressed with GABAergic neurons in RP3V[12,43,63] and MEA,[64,65] glutamatergic neurons in Arc[63,66,67] and RP3V,[63,67] dynorphin/neurokinin-containing neurons in Arc[34,36] and RP3V,[36] estrogen and androgen receptive neurons of RP3V and Arc,[20,23,65] ours is the first report to systematically characterize the chemotypes of these KP populations in the mouse brain to form a basis for the extrapolation of future experiments examining behavioral, as well as endocrine regulation by kisspeptin.

4.1 | Physiological implications of seven KP mRNA-expressing populations throughout brain

For the two major KP hypothalamic groups, namely the rostro-periventricular group (KP^{RP3V}) and the arcuate hypothalamic group (KP^{Arc}), our observations mainly concur with reports in the literature. However, using the highly sensitive "Brown kit" RNAscope method, we 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 express KP mRNA and translate it to peptide in dendrites, allowing neurons to rapidly respond to synaptic signals with spatial precision, thereby enhancing synaptic plasticity.[68–71]

The R3PV kisspeptin neurons play a key role in the positive feedback of estrogen, which is critical for the stimulation of gonadotropin-releasing hormone (GnRH) resulting in the preovulatory surge of luteinizing hormone (LH) which culminates in ovulation. 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.[72–74] We demonstrated that the KP^{RP3V} population co-expresses the *Slc32a1*, *Esr1*, *Ar*, and *Pdyn* RNAs. In agreement with the majority of previous reports characterizing mRNA expression in this region, we observed sexual dimorphism and the effects of gonadectomy.[20,23,75,76] We observed a higher number of KP-expressing 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 particularly significant 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.

Arc kisspeptin neurons (KP^{Arc}) mediate the negative feedback regulation of GnRH secretion. High estrogen levels suppress Kiss1 expression in these neurons and downstream stimulation of GnRH neurons culminates in increased LH pulsatility and amplitude necessary for normal reproductive function.[72,77–79] In the arcuate hypothalamic group, the KP neurons co-express *Slc17a6*, *Esr1*, *Ar*, *Pdyn*, and *Tac2*, and have been extensively characterized as "KNDy" neurons.[80] 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 (20.3%) than in proestrous females (6.4%). It has been classically considered that the plasma concentration of estradiol is highest during proestrus,[81] however, a recent study in mice showed that although in proestrus the estradiol levels do have a peak, it is during diestrus that estradiol concentrations reach their maximum.[82] Thus, in addition to the regulation of dendritic Kiss1 mRNA in males compared with females, there may also be differences during the estrous cycle in females as well. Gonadectomy caused a robust increase in dendritic mRNA labeling within *Kiss1*-expressing cells, of 69.8% in males and 79.3% in females. Thus, *Kiss1* dendritic expression in both KP^{RP3V} and KP^{Arc} represents a new arena of KP cellular physiology exploration, albeit requiring correlation with sites of 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,[24,75] while others indicate a higher number of *Kiss1*-expressing neurons in males[20,23] or in females.[76] These discrepancies may stem from factors such as threshold criteria for *Kiss1* positivity, the dense neuronal packing within the region, and/or the varied distribution along the rostro-caudal 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 (DMH) was identified and characterized at the mRNA level in the present report. Previous immunohistochemical studies[22,61,83] and *Kiss1*-CRE mice[59,63] showed the presence 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 antibodies cross-reacting with both peptides.[77] However, we confirm here the existence of these dispersed KPergic neurons by RNAscope in situ hybridization. Since the mRNA probe targets a segment 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). 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 the horse[84] and indirectly by GFP expression in *Kiss1*-GFP transgenic mice.[85] The authors of the study in the 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 GFP 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.[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 MeAPD have been studied most extensively.[17,24,33,57,65,83] This region is involved in processing olfactory stimuli and influencing mating behavior and reproductive functions.[64] 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 via sex-hormone signaling pathways. The chemoanatomical markers identified in these MePD cells are congruent with a recent study in female mice where RiboTag/RNA-seq was used to demonstrate *Pdyn*, *Ar*, and *Esr1* expression within medial amygdala tissue from *Kiss1*-Cre mice.[65] The male-specific reduction in KP mRNA expression in KP^{MePD} neurons following gonadectomy, reported in this study, supports the putative 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 interconnected regions such as the hippocampus and cingulate cortex, which are crucial to emotional regulation, sexual response, and bonding.[87]

The sixth KP population was widely distributed in the strio-septo-hypothalamic region, centered in the septo-hypothalamic nucleus, as a sparse spatial continuum (see Figure 1, F panels for a parasagittal view and Figure S1 for coronal views at bregma 0.38 mm and 0.02 mm according to Paxinos and Franklin.[53]) We referred to this population in our study as KP^{SHy}. The cells distributed in the strio-septo-hypothalamic subfield shared the same molecular signature among them and with KP^{MePD} neurons, expressing both VGAT and sex steroid receptors (*Ar* and *Esr1*), suggesting a related embryonic origin. Some authors have reported the existence of a rather large population of *Kiss1*-expressing neurons in the lateral septal nucleus,[24,33,59,60] upon various genetic or pharmacological/hormonal manipulations. We did not identify a prominent *Kiss1*-expressing population in this region in our study, using the SISH method in wild-type, intact mice without additional pharmacological or hormonal manipulations. However, we report some sporadic *Kiss1*-expressing neurons detected in some sections in the region of the lateral septal nucleus bordering the antero-medial bed nucleus of the stria terminalis (BSTam, Figure S1). These discrepant results suggest the existence of a septal neuronal population with latent potential for *Kiss1* expression, under specific circumstances or physiological challenges (such as GDX and hormone replacement[33]) or, in the case of detection using *Kiss1* promoter-Cre mice, reflecting developmental *Kiss1* gene expression in this region.

Considering the role of the septum and BST in emotional processing, stress response, motivation, and reward-seeking behavior, as well as their connections with the hypothalamus and other limbic structures,[88–91] 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 in a sexually dimorphic manner, as this was 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*, 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.[61,62] 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 the 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 MeAPD and SHy may 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 impacts of these KP neurons could provide new insights into the pleiotropic roles of KP signaling in brain function beyond hypothalamic reproductive control.

4.2 | Distinct responsiveness to gonadectomy of the seven KPergic populations

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 provides a molecular explanation for the distinction among KP neuronal populations with respect to changes in KP expression upon gonadectomy. Four patterns of alteration to 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.[80] Confirmation of up-regulation of KP expression by GNX in the 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.[92] The third pattern of alteration by gonadectomy was down-regulation in males but no significant effect in females, 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 undetectable 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.

Previous studies using immunohistochemistry and classical in-situ hybridization have reported that the MePD and BST regions have a higher number of *Kiss1* neurons in males than in females, and that in gonadectomized animals, *Kiss1* expression is almost undetectable.[18,24,33] Additionally, it has been reported that the effects of sex steroids are mediated mainly through signaling via estrogen receptor alpha.[18] However, in the same study, the authors demonstrated that while estradiol and testosterone increased the Kiss1 mRNA silver grains per cell, the effect of testosterone was significantly higher than that of estradiol, suggesting an additional role for androgen receptor (AR) signaling. Our results, using a different method of mRNA detection with greater sensitivity, confirm the results of Kim et al. in male mice.[18] In any case, a physiological explanation for a lesser effect of gonadectomy on Kiss1 mRNA expression in this/these nucleus/nuclei in female mice might be that loss of estrogen removes tonic inhibition on ACTH secretion,[93–96] which in females creates a compensatory increase in *Kiss1* mRNA in these nuclei by enhanced adrenocortical androgen production.[97,98]

It is intriguing that of the two major KP cell groups in the hypothalamus, those in the RP3V and in the Arc are oppositely regulated by gonadectomy, are of opposite chemotype (GABAergic and glutamatergic, respectively), and are 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 ultimately impacting GnRH function.[99] 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.[100] How the post-synaptic effects are modulated by the 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 kernel role of kisspeptin in human sexually related behavior has been provided by clinical studies of kisspeptin administration in men and women.[87,101] However, except for some scattered reports,[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,[42] 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.

AUTHOR CONTRIBUTIONS

Vito S. Hernández: Conceptualization; investigation; funding acquisition; writing – original draft; methodology; validation; visualization; writing – review and editing; resources. **Mario A. Zetter:** Conceptualization; investigation; validation; visualization; writing – review and editing. **Oscar R. Hernández-Pérez:** Investigation; writing – review and editing; validation. **Rafael Hernández-González:** Investigation; methodology; validation; resources; writing – review and editing. **Ignacio Camacho-Arroyo:** Writing – review and editing; validation; investigation; supervision; resources. **Robert P. Millar:** Conceptualization; writing – review and editing; resources; supervision. **Lee E. Eiden:** Conceptualization; investigation; funding acquisition; writing – original draft; writing – review and editing; validation; resources. **Limei Zhang:** Conceptualization; investigation; funding acquisition; writing – original draft; methodology; validation; visualization; writing – review and editing; project administration; resources; supervision.

ACKNOWLEDGEMENTS

The research leading to these results received funding from the National Autonomous University of Mexico, under Grant Agreement UNAM-PAPIIT-IG200121 (LZ), Mexican Nacional Council for Humanity, Science and Technology (CONAHCYT), under grant agreement CF-2023-G-243 (LZ), NIMH-IR, NIH, under Grant Agreement MH002386 (LEE). The investigators of this study have been supported by the following fellowships: sabbatical year fellowship from the PASPA program of the Dirección General de Personal Académico (DGAPA) of the Universidad Nacional Autónoma de México (UNAM) (LZ, VSH); Mexican CONAHCYT sabbatical fellowship (LZ); Fulbright-García Robles Fellowship (VSH); DGAPA-UNAM POSDOC program (MAZ) and PREI program for a sabbatical research stay (RPM) in LZ's lab in UNAM, Mexico. RM thanks the University of Pretoria for sabbatical leave and the National Research Foundation and

Medical Research Council of South Africa for research grant support. Open access funding provided by UNAM.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Lee E. Eiden https://orcid.org/0000-0001-7524-944X
Limei Zhang https://orcid.org/0000-0002-7422-5136

REFERENCES

1. Kotani M, Detheux M, Vandenbogaerde A, et al. The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. *J Biol Chem*. 2001; 276(37):34631-34636.
2. Makri A, Pissimissis N, Lembessis P, Polychronakos C, Koutsilieris M. The kisspeptin (KiSS-1)/GPR54 system in cancer biology. *Cancer Treat Rev*. 2008;34(8):682-692.
3. Lee JH, Miele ME, Hicks DJ, et al. KiSS-1, a novel human malignant melanoma metastasis-suppressor gene. *J Natl Cancer Inst*. 1996; 88(23):1731-1737.
4. Lee DK, Nguyen T, O'Neill GP, et al. Discovery of a receptor related to the galanin receptors. *FEBS Lett*. 1999;446(1):103-107.
5. Dungan HM, Clifton DK, Steiner RA. Minireview: kisspeptin neurons as central processors in the regulation of gonadotropin-releasing hormone secretion. *Endocrinology*. 2006;147(3):1154-1158.
6. Gottsch ML, Cunningham MJ, Smith JT, et al. A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. *Endocrinology*. 2004;145(9):4073-4077.
7. Irwig MS, Fraley GS, Smith JT, et al. Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat. *Neuroendocrinology*. 2004;80(4):264-272.
8. Seminara SB, Messager S, Chatzidaki EE, et al. The GPR54 gene as a regulator of puberty. *N Engl J Med*. 2003;349(17):1614-1627.
9. de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc Natl Acad Sci U S A*. 2003;100(19):10972-10976.
10. Clarke H, Dhillo WS, Jayasena CN. Comprehensive review on Kisspeptin and its role in reproductive disorders. *Endocrinol Metab Seoul*. 2015;30(2):124-141.
11. Lehman MN, Coolen LM, Steiner RA, et al. The 3(rd) world conference on Kisspeptin, "Kisspeptin 2017: brain and beyond": unresolved questions, challenges and future directions for the field. *J Neuroendocrinol*. 2018;30:e12600.
12. Piet R, Kalil B, McLennan T, Porteous R, Czieselsky K, Herbison AE. Dominant neuropeptide cotransmission in Kisspeptin-GABA regulation of GnRH neuron firing driving ovulation. *J Neurosci*. 2018; 38(28):6310-6322.
13. Liu X, Yeo SH, McQuillan HJ, et al. Highly redundant neuropeptide volume co-transmission underlying episodic activation of the GnRH neuron dendron. *Elife*. 2021;10:e62455. doi:10.7554/elife.62455
14. Liu X, Porteous R, Herbison AE. Robust GABAergic regulation of the GnRH neuron distal dendron. *Endocrinology*. 2022;164(1):bqac194. doi:10.1210/endocr/bqac194
15. Mills EG, Yang L, Abbara A, Dhillo WS, Comninos AN. Current perspectives on Kisspeptins role in behaviour. *Front Endocrinol Lausanne*. 2022;13:13928143. doi:10.3389/fendo.2022.928143
16. Lopez-Ojeda W, Hurley RA. Kisspeptin in the limbic system: new insights into its neuromodulatory roles. *J Neuropsychiatry Clin Neurosci*. 2022;34(3):190-195.
17. Pineda R, Plaisier F, Millar RP, Ludwig M. Amygdala Kisspeptin neurons: putative mediators of olfactory control of the gonadotropic axis. *Neuroendocrinology*. 2017;104(3):223-238.
18. Kim J, Semaan SJ, Clifton DK, Steiner RA, Dhamija S, Kauffman AS. Regulation of Kiss1 expression by sex steroids in the amygdala of the rat and mouse. *Endocrinology*. 2011;152(5):2020-2030.
19. Gresham R, Li S, Adekunbi DA, Hu M, Li XF, O'Byrne KT. Kisspeptin in the medial amygdala and sexual behavior in male rats. *Neurosci Lett*. 2016;627:13-17.
20. Smith JT, Cunningham MJ, Rissman EF, Clifton DK, Steiner RA. Regulation of Kiss1 gene expression in the brain of the female mouse. *Endocrinology*. 2005;146(9):3686-3692.
21. Lehman MN, Merkley CM, Coolen LM, Goodman RL. Anatomy of the kisspeptin neural network in mammals. *Brain Res*. 2010;1364: 136490-102. doi:10.1016/j.brainres.2010.09.020
22. Clarkson J, Herbison AE. Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons. *Endocrinology*. 2006; 147(12):5817-5825.
23. Smith JT, Dungan HM, Stoll EA, et al. Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse. *Endocrinology*. 2005;146(7):2976-2984.
24. Di Giorgio NP, Semaan SJ, Kim J, et al. Impaired GABAB receptor signaling dramatically up-regulates Kiss1 expression selectively in nonhypothalamic brain regions of adult but not prepubertal mice. *Endocrinology*. 2014;155(3):1033-1044.
25. Franceschini I, Yeo SH, Beltramo M, et al. Immunohistochemical evidence for the presence of various kisspeptin isoforms in the mammalian brain. *J Neuroendocrinol*. 2013;25(9):839-851.
26. Lehman MN, Hileman SM, Goodman RL. Neuroanatomy of the kisspeptin signaling system in mammals: comparative and developmental aspects. *Adv Exp Med Biol*. 2013;784:27-62.
27. Hudson AD, Kauffman AS. Metabolic actions of kisspeptin signaling: effects on body weight, energy expenditure, and feeding. *Pharmacol Ther*. 2022;231:231107974. doi:10.1016/j.pharmthera.2021.107974
28. Izzi-Engbeaya C, Dhillo WS. Emerging roles for kisspeptin in metabolism. *J Physiol*. 2022;600(5):1079-1088.
29. Aerts EG, Harlow K, Griesgraber MJ, et al. Kisspeptin, neurokinin B, and dynorphin expression during pubertal development in female sheep. *Biology Basel*. 2021;10(10):988. doi:10.3390/biology10100988
30. Clarke SA, Dhillo WS. Kisspeptin across the human lifespan: evidence from animal studies and beyond. *J Endocrinol*. 2016;229(3): R83-R98.
31. Pinilla L, Aguilar E, Dieguez C, Millar RP, Tena-Sempere M. Kisspeptins and reproduction: physiological roles and regulatory mechanisms. *Physiol Rev*. 2012;92(3):1235-1316.
32. Huang Y, Liu Q, Huang G, Wen J, Chen G. Hypothalamic Kisspeptin neurons regulates energy metabolism and reproduction under chronic stress. *Front Endocrinol Lausanne*. 2022;13:13844397. doi:10.3389/fendo.2022.844397
33. Stephens SBZ, Di Giorgio NP, Liaw RB, et al. Estradiol-dependent and -independent stimulation of Kiss1 expression in the amygdala, BNST, and lateral septum of mice. *Endocrinology*. 2018;159(9):3389-3402.
34. Goodman RL, Lehman MN, Smith JT, et al. Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin A and neurokinin B. *Endocrinology*. 2007;148(12):5752-5760.
35. Moore AM, Coolen LM, Porter DT, Goodman RL, Lehman MN. KNDy cells revisited. *Endocrinology*. 2018;159(9):3219-3234.
36. Navarro VM, Gottsch ML, Chavkin C, Okamura H, Clifton DK, Steiner RA. Regulation of gonadotropin-releasing hormone secretion

by kisspeptin/dynorphin/neurokinin B neurons in the arcuate nucleus of the mouse. *J Neurosci*. 2009;29(38):11859-11866.
37. Skrapits K, Borsay BA, Herczeg L, Ciofi P, Liposits Z, Hrabovszky E. Neuropeptide co-expression in hypothalamic kisspeptin neurons of laboratory animals and the human. *Front Neurosci*. 2015;9:29.
38. Zhang C, Roepke TA, Kelly MJ, Ronnekleiv OK. Kisspeptin depolarizes gonadotropin-releasing hormone neurons through activation of TRPC-like cationic channels. *J Neurosci*. 2008;28(17):4423-4434.
39. Liu X, Lee K, Herbison AE. Kisspeptin excites gonadotropin-releasing hormone neurons through a phospholipase C/calcium-dependent pathway regulating multiple ion channels. *Endocrinology*. 2008; 149(9):4605-4614.
40. Wes PD, Chevesich J, Jeromin A, Rosenberg C, Stetten G, Montell C. TRPC1, a human homolog of a *Drosophila* store-operated channel. *Proc Natl Acad Sci U S A*. 1995;92(21):9652-9656.
41. Zhu X, Chu PB, Peyton M, Birnbaumer L. Molecular cloning of a widely expressed human homologue for the *Drosophila* trp gene. *FEBS Lett*. 1995;373(3):193-198.
42. Liu X, Herbison AE. Kisspeptin regulation of neuronal activity throughout the central nervous system. *Endocrinol Metab Seoul*. 2016;31(2):193-205.
43. Ronnekleiv OK, Qiu J, Kelly MJ. Hypothalamic Kisspeptin neurons and the control of homeostasis. *Endocrinology*. 2022;163(2):1-11. doi:10.1210/endocr/bqab253
44. Iremonger KJ, Porteous R, Herbison AE. Spike and neuropeptide-dependent mechanisms control GnRH neuron nerve terminal Ca(2+) over diverse time scales. *J Neurosci*. 2017;37(12):3342-3351.
45. Pielecka-Fortuna J, Chu Z, Moenter SM. Kisspeptin acts directly and indirectly to increase gonadotropin-releasing hormone neuron activity and its effects are modulated by estradiol. *Endocrinology*. 2008; 149(4):1979-1986.
46. Leon S, Barroso A, Vazquez MJ, et al. Direct actions of Kisspeptins on GnRH neurons permit attainment of fertility but are insufficient to fully preserve gonadotropic axis activity. *Sci Rep*. 2016;6:19206.
47. Cora MC, Kooistra L, Travlos G. Vaginal cytology of the laboratory rat and mouse: review and criteria for the staging of the estrous cycle using stained vaginal smears. *Toxicol Pathol*. 2015;43(6): 776-793.
48. McLean AC, Valenzuela N, Fai S, Bennett SA. Performing vaginal lavage, crystal violet staining, and vaginal cytological evaluation for mouse estrous cycle staging identification. *J Vis Exp*. 2012;(67): e4389. doi:10.3791/4389
49. Zhang L, Hernández VS, Zetter MA, et al. Kisspeptin cell-type and fiber projection analysis suggests its potential role in central sensorial processing and behavioral state control. *J Neuroendocrinol*. 2025;e70007. doi:10.1111/jne.70007
50. Zhang L, Hernandez VS, Gerfen CR, et al. Behavioral role of PACAP signaling reflects its selective distribution in glutamatergic and GABAergic neuronal subpopulations. *Elife*. 2021;10:10. doi:10.7554/elife.61718
51. Zhang L, Hernandez VS, Zetter MA, Eiden LE. VGLUT-VGAT expression delineates functionally specialised populations of vasopressin-containing neurones including a glutamatergic perforant path-projecting cell group to the hippocampus in rat and mouse brain. *J Neuroendocrinol*. 2020;32(4):e12831.
52. Zhang L, Hernandez VS, Swinny JD, et al. A GABAergic cell type in the lateral habenula links hypothalamic homeostatic and midbrain motivation circuits with sex steroid signaling. *Transl Psychiatry*. 2018;8(1):50.
53. Paxinos G, Franklin KBJ. *Paxinos and Franklin's the Mouse Brain in Stereotaxic Coordinates*. Elsevier Science; 2012.
54. Moore AM, Coolen LM, Lehman MN. Kisspeptin/neurokinin B/-dynorphin (KNDy) cells as integrators of diverse internal and external cues: evidence from viral-based monosynaptic tract-tracing in mice. *Sci Rep*. 2019;9(1):14768.
55. Stephens SBZ, Kauffman AS. Regulation and possible functions of Kisspeptin in the medial amygdala. *Front Endocrinol Lausanne*. 2017; 8:191. doi:10.3389/fendo.2017.00191
56. Franklin KBJ, Paxinos G. *The Mouse Brain in Stereotaxic Coordinates*. Elsevier; 2008.
57. Xu Z, Kaga S, Mochiduki A, et al. Immunocytochemical localization of kisspeptin neurons in the rat forebrain with special reference to sexual dimorphism and interaction with GnRH neurons. *Endocr J*. 2012;59(2):161-171.
58. Yamada K, Nagae M, Mano T, et al. Sex difference in developmental changes in visualized Kiss1 neurons in newly generated Kiss1-Cre rats. *J Reprod Dev*. 2023;69(5):227-238.
59. Yeo SH, Kyle V, Morris PG, et al. Visualisation of Kiss1 Neurone distribution using a Kiss1-CRE transgenic mouse. *J Neuroendocrinol*. 2016;28(11). doi:10.1111/jne.12435
60. Szentkiralyi-Toth S, Gocz B, Takacs S, et al. Estrogen-regulated lateral septal kisspeptin neurons abundantly project to GnRH neurons and the hypothalamic supramammillary nucleus. *J Neurosci*. 2025;45: e1307242024.
61. Brailoiu GC, Dun SL, Ohsawa M, et al. KiSS-1 expression and metastin-like immunoreactivity in the rat brain. *J Comp Neurol*. 2005; 481(3):314-329.
62. Dun SL, Brailoiu GC, Parsons A, et al. Metastin-like immunoreactivity in the rat medulla oblongata and spinal cord. *Neurosci Lett*. 2003; 335(3):197-201.
63. Cravo RM, Margatho LO, Osborne-Lawrence S, et al. Characterization of Kiss1 neurons using transgenic mouse models. *Neuroscience*. 2011;173:37-56.
64. Aggarwal S, Tang C, Sing K, Kim HW, Millar RP, Tello JA. Medial amygdala Kiss1 neurons mediate female pheromone stimulation of luteinizing hormone in male mice. *Neuroendocrinology*. 2019;108(3): 172-189.
65. Hatcher KM, Costanza L, Kauffman AS, Stephens SBZ. The molecular phenotype of kisspeptin neurons in the medial amygdala of female mice. *Front Endocrinol Lausanne*. 2023;14:141093592. doi: 10.3389/fendo.2023.1093592
66. Nestor CC, Qiu J, Padilla SL, et al. Optogenetic stimulation of arcuate nucleus Kiss1 neurons reveals a steroid-dependent glutamatergic input to POMC and AgRP neurons in male mice. *Mol Endocrinol*. 2016;30(6):630-644.
67. Cheong RY, Czieselsky K, Porteous R, Herbison AE. Expression of ESR1 in glutamatergic and GABAergic neurons is essential for Normal puberty onset, estrogen feedback, and fertility in female mice. *J Neurosci*. 2015;35(43):14533-14543.
68. Bramham CR, Wells DG. Dendritic mRNA: transport, translation and function. *Nat Rev Neurosci*. 2007;8(10):776-789.
69. Holt CE, Martin KC, Schuman EM. Local translation in neurons: visualization and function. *Nat Struct Mol Biol*. 2019;26(7):557-566.
70. Kaneko M, Xie Y, An JJ, Stryker MP, Xu B. Dendritic BDNF synthesis is required for late-phase spine maturation and recovery of cortical responses following sensory deprivation. *J Neurosci*. 2012;32(14): 4790-4802.
71. Liao GY, An JJ, Gharami K, et al. Dendritically targeted Bdnf mRNA is essential for energy balance and response to leptin. *Nat Med*. 2012;18(4):564-571.
72. Gocz B, Takacs S, Skrapits K, et al. Estrogen differentially regulates transcriptional landscapes of preoptic and arcuate kisspeptin neuron populations. *Front Endocrinol Lausanne*. 2022;13:960769.
73. Kumar D, Candlish M, Periasamy V, Avcu N, Mayer C, Boehm U. Specialized subpopulations of kisspeptin neurons communicate with GnRH neurons in female mice. *Endocrinology*. 2015;156(1): 32-38.
74. Stevenson H, Bartram S, Charalambides MM, et al. Kisspeptin-neuron control of LH pulsatility and ovulation. *Front Endocrinol Lausanne*. 2022;13:13951938. doi:10.3389/fendo.2022.951938

75. Kauffman AS. Gonadal and nongonadal regulation of sex differences in hypothalamic Kiss1 neurones. *J Neuroendocrinol*. 2010;22(7): 682-691.
76. Takumi K, Iijima N, Iwata K, Higo S, Ozawa H. The effects of gonadal steroid manipulation on the expression of Kiss1 mRNA in rat arcuate nucleus during postnatal development. *J Physiol Sci*. 2012;62(6): 453-460.
77. Oakley AE, Clifton DK, Steiner RA. Kisspeptin signaling in the brain. *Endocr Rev*. 2009;30(6):713-743.
78. Hrabovszky E. Neuroanatomy of the human hypothalamic kisspeptin system. *Neuroendocrinology*. 2014;99(1):33-48.
79. Mostari P, Ieda N, Deura C, et al. Dynorphin-kappa opioid receptor signaling partly mediates estrogen negative feedback effect on LH pulses in female rats. *J Reprod Dev*. 2013;59(3):266-272.
80. Lehman MN, Coolen LM, Goodman RL. Minireview: kisspeptin/neurokinin B/dynorphin (KNDy) cells of the arcuate nucleus: a central node in the control of gonadotropin-releasing hormone secretion. *Endocrinology*. 2010;151(8):3479-3489.
81. Smith MS, Freeman ME, Neill JD. The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. *Endocrinology*. 1975;96(1): 219-226.
82. Wall EG, Desai R, Khant Aung Z, et al. Unexpected plasma gonadal steroid and prolactin levels across the mouse estrous cycle. *Endocrinology*. 2023;164(6):bqad070. doi:10.1210/endocr/bqad070
83. Clarkson J, de d'Anglemont Tassigny X, Colledge WH, Caraty A, Herbison AE. Distribution of kisspeptin neurones in the adult female mouse brain. *J Neuroendocrinol*. 2009;21(8):673-682.
84. Decourt C, Tillet Y, Caraty A, Franceschini I, Briant C. Kisspeptin immunoreactive neurons in the equine hypothalamus interactions with GnRH neuronal system. *J Chem Neuroanat*. 2008;36(3–4): 131-137.
85. de Croft S, Piet R, Mayer C, Mai O, Boehm U, Herbison AE. Spontaneous kisspeptin neuron firing in the adult mouse reveals marked sex and brain region differences but no support for a direct role in negative feedback. *Endocrinology*. 2012;153(11):5384-5393.
86. Smith AS, Williams ASK, Cymerblit-Sabba A, Song J, Young WS. Targeted activation of the hippocampal CA2 area strongly enhances social memory. *Mol Psychiatry*. 2016;21(8):1137-1144.
87. Comninos AN, Wall MB, Demetriou L, et al. Kisspeptin modulates sexual and emotional brain processing in humans. *J Clin Invest*. 2017; 127(2):709-719.
88. Besnard A, Leroy F. Top-down regulation of motivated behaviors via lateral septum sub-circuits. *Mol Psychiatry*. 2022;27(8):3119-3128.
89. Ch'ng S, Fu J, Brown RM, McDougall SJ, Lawrence AJ. The intersection of stress and reward: BNST modulation of aversive and appetitive states. *Prog Neuropsychopharmacol Biol Psychiatry*. 2018; 87(Pt A):108-125.
90. Lebow MA, Chen A. Overshadowed by the amygdala: the bed nucleus of the stria terminalis emerges as key to psychiatric disorders. *Mol Psychiatry*. 2016;21(4):450-463.
91. Maita I, Bazer A, Blackford JU, Samuels BA. Functional anatomy of the bed nucleus of the stria terminalis-hypothalamus neural circuitry: implications for valence surveillance, addiction, feeding, and social behaviors. *Handb Clin Neurol*. 2021;179:403-418.
92. Saldanha CJ, Walters BJ, Fraley GS. Neurons that co-localize aromatase- and kisspeptin-like immunoreactivity may regulate the HPG axis of the Mallard drake (*Anas platyrhynchos*). *Gen Comp Endocrinol*. 2010;166(3):606-613.
93. Lund TD, Rovis T, Chung WC, Handa RJ. Novel actions of estrogen receptor-beta on anxiety-related behaviors. *Endocrinology*. 2005; 146(2):797-807.
94. Young EA, Altemus M, Parkison V, Shastry S. Effects of estrogen antagonists and agonists on the ACTH response to restraint stress in female rats. *Neuropsychopharmacology*. 2001;25(6):881-891.
95. Handa RJ, Weiser MJ. Gonadal steroid hormones and the hypothalamo-pituitary-adrenal axis. *Front Neuroendocrinol*. 2014; 35(2):197-220.
96. Weiser MJ, Handa RJ. Estrogen impairs glucocorticoid dependent negative feedback on the hypothalamic-pituitary-adrenal axis via estrogen receptor alpha within the hypothalamus. *Neuroscience*. 2009;159(2):883-895.
97. Ganong WF, Alpert LC, Lee TC. ACTH and the regulation of adrenocortical secretion. *N Engl J Med*. 1974;290(18):1006-1011.
98. Burger HG. Androgen production in women. *Fertil Steril*. 2002;77-(Suppl 4):S3-S5.
99. Yip SH, Boehm U, Herbison AE, Campbell RE. Conditional viral tract tracing delineates the projections of the distinct Kisspeptin neuron populations to gonadotropin-releasing hormone (GnRH) neurons in the mouse. *Endocrinology*. 2015;156(7):2582-2594.
100. Stincic TL, Qiu J, Connors AM, Kelly MJ, Ronnekleiv OK. Arcuate and preoptic Kisspeptin neurons exhibit differential projections to hypothalamic nuclei and exert opposite postsynaptic effects on hypothalamic paraventricular and dorsomedial nuclei in the female mouse. *eNeuro*. 2021;8(4):ENEURO.0093.
101. Petrine JCP, Franci CR, Del Bianco-Borges B. Leptin actions through the nitrergic system to modulate the hypothalamic expression of the kiss1 mRNA in the female rat. *Brain Res*. 2020;1728:146574.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Hernández VS, Zetter MA, Hernández-Pérez OR, et al. Comprehensive chemoanatomical mapping, and the gonadal regulation, of seven kisspeptin neuronal populations in the mouse brain. *J Neuroendocrinol*. 2025;37(5):e70019. doi:10.1111/jne.70019