Conditional Viral Tract Tracing Delineates the Projections of the Distinct Kisspeptin Neuron Populations to Gonadotropin-Releasing Hormone (GnRH) Neurons in the Mouse

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Kisspeptin neurons play an essential role in the regulation of fertility through direct regulation of the GnRH neurons. However, the relative contributions of the two functionally distinct kisspeptin neuron subpopulations to this critical regulation are not fully understood. Here we analyzed the specific projection patterns of kisspeptin neurons originating from either the rostral periventricular nucleus of the third ventricle (RP3V) or the arcuate nucleus (ARN) using a cell-specific, viral-mediated tract-tracing approach. We stereotaxically injected a Cre-dependent recombinant adenovirus encoding farnesylated enhanced green fluorescent protein into the ARN or RP3V of adult male and female mice expressing Cre recombinase in kisspeptin neurons. Fibers from ARN kisspeptin neurons projected widely; however, we did not find any evidence for direct contact with GnRH neuron somata or proximal dendrites in either sex. In contrast, we identified RP3V kisspeptin fibers in close contact with GnRH neuron somata and dendrites in both sexes. Fibers originating from both the RP3V and ARN were observed in close contact with distal GnRH neuron processes in the ARN and in the lateral and internal aspects of the median eminence. Furthermore, GnRH nerve terminals were found in close contact with the proximal dendrites of ARN kisspeptin neurons in the ARN, and ARN kisspeptin fibers were found contacting RP3V kisspeptin neurons in both sexes. Together these data delineate selective zones of kisspeptin neuron inputs to GnRH neurons and demonstrate complex interconnections between the distinct kisspeptin populations and GnRH neurons. (***Endocrinology*** **156: 2582–2594, 2015)**

Acknowledged as a critical regulator of puberty and reproductive function, the neuropeptide kisspeptin potently drives the activity of GnRH neurons (1, 2). Evidence for two distinct populations of kisspeptin neurons, in the preoptic area and the arcuate nucleus (ARN), exists in all mammalian species investigated, including the human (3–6). However, the relative roles that the anatomically distinct populations of kisspeptin neurons play in the regulation of GnRH neuron function and fertility remain to be clearly defined.

The kisspeptin neurons located in the ARN in rodents are highly sexually differentiated, with females possessing approximately 10 times the number of kisspeptin neurons as males (7), and are strongly implicated in the generation of the preovulatory surge (8, 9). It has been appreciated for some time now that estradiol exerts opposite effects in the two populations of kisspeptin neurons, elevating *Kiss1* mRNA in the rostral periventricular nucleus of the third ventricle (RP3V) and decreasing *Kiss1* mRNA in the ARN (10), suggesting that the disparate kisspeptin populations play distinct roles in the regulation of GnRH neuron activity and secretion. This has led to the widely held hypothesis that kisspeptin neurons in the RP3V are responsible for estradiol-positive feedback and the surge mode of

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Abbreviations: AdV-EGFPf, adenoviral vector EGFPf; AHA, anterior hypothalamic area; ARN, arcuate nucleus; AVPV, anteroventral periventricular nucleus; DMH, dorsomedial hypothalamic nucleus; EGFP, enhanced green fluorescent protein; EGFPf, farnesylated EGFP; GFP, green fluorescent protein; IHC, immunohistochemistry; LHA, lateral hypothalamic area; LPZ, lateral palisade zone; MS, medial septum; rPOA, rostral preoptic area; RP3V, rostral periventricular nucleus of the third ventricle; TBS, Tris-buffered saline.

GnRH secretion (11–14). In contrast, the role of the ARN kisspeptin population is much less certain, and it has been suggested to have various roles in puberty onset (15), estrogen-negative feedback (16), and GnRH pulsatility (17). It remains to be fully determined how these distinct modes of activation are achieved.

Kisspeptin regulation of GnRH neuron activity and secretion is considered to be predominantly direct. GnRH neurons express the kisspeptin receptor, GPR54, and GnRH neuron-specific expression of GPR54 is required for fertility (18). Kisspeptin-positive fibers make contact and synapse onto GnRH neurons (7, 19–21) and direct synaptic inputs onto GnRH neurons are supported by functional studies (1, 22). Studies have found that kisspeptin can influence the activity of GnRH neurons at the level of the soma and dendrites (1, 22, 23), but also at the very distal terminals of GnRH neurons in the region of the median eminence (24–26). Understanding the relative contributions that the functionally and anatomically distinct kisspeptin populations make to direct GnRH neuron innervation at various levels of the cell is important for understanding how kisspeptin regulates GnRH neuron function and fertility.

To date, the distinct projection patterns of kisspeptin neurons have been identified using either classical neuronal tract tracing coupled with immunohistochemistry (IHC) for kisspeptin, or multiple-label IHC to identify neuropeptides uniquely expressed within the fibers of each distinct population (27–31). Evidence to date suggests that most kisspeptin innervation to GnRH neuron cell bodies and proximal dendrites most likely originates predominantly from RP3V neurons, identified by their coexpression of tyrosine hydroxylase or galanin (30, 31). However, the location and degree of direct innervation from ARN kisspeptin neurons to GnRH neurons remains less clear. Dense ARN kisspeptin fibers, identified by their coexpression with neurokinin B, are found throughout the ARN and internal zone of the median eminence (31, 32), suggesting that ARN kisspeptin projections may regulate the distal portions of GnRH neuron dendrites and terminals.

In the present study, we have used a viral-mediated, transgenic tract-tracing method to definitively identify the projection patterns of the distinct kisspeptin populations. Using a Cre-lox approach and stereotaxic delivery of a conditional transgene expressing a farnesylated enhanced green fluorescent protein (EGFPf) (33), we have targeted kisspeptin neurons in either the RP3V or ARN. The hydrophobic nature of the farnesyl group renders the trafficking of the enhanced green fluorescent protein (EGFP) to the cell membrane, effectively labeling the distal axonal projections that can be subsequently imaged in association with the GnRH neurons.

Materials and Methods

Animals

Adult male and female kisspeptin-internal ribosome entry site-Cre$^{+/-}$ (Kiss-Cre) mice (15, 34) (age 2–4 mo) were housed under conditions of controlled temperature and a 12-hour light, 12-hour dark cycle (lights on at 6:00 AM) with ad libitum access to food and water. All experimental procedures were approved by the University of Otago Animal Welfare and Ethics Committee.

Cell-specific tract tracing

A previously described recombinant adenoviral vector containing a cytomegalovirus promoter upstream of a transcription-blocking cassette, followed by sequences encoding farnesylated EGFP (AdV-EGFPf; 5.1×10^{11} pfu/mL stock; a kind gift of Professor Martin G. Myers Jr (University of Michigan, Ann Arbor, Michigan) was used to mediate the expression of EGFPf exclusively in Cre-expressing neurons (35, 36).

Unilateral stereotaxic injections of AdV-EGFPf were made into male and freely cycling female Kiss-Cre mice, targeting either the ARN or RP3V. Two wild-type (male) mice received injections into the RP3V to determine the Cre-dependent expression of the transgene. Under 2% isoflurane anesthesia, mice were placed in a stereotaxic apparatus. The skull was exposed and coordinates were recorded from bregma. ARN injections were targeted to −1.1 mm posterior to bregma, −0.2 mm lateral to midline, and −6.0 mm ventral to dura (according to the atlas of Paxinos and Franklin). RP3V injections were targeted to +0.6 mm posterior to bregma, +0.2 mm lateral to midline, and −5.0 mm ventral to dura. A Hamilton syringe loaded with 300 nL of AdV-EGFPf (5.1×10^{11} pfu/mL) was lowered into place and injected freehand at a rate of 100 nL/min. Seven days after the injections, the animals were anesthetized with an overdose of pentobarbital (3 mg per 100 μL) and transcardially perfused with 20 mL of 4% paraformaldehyde. Brains were removed, postfixed in 4% paraformaldehyde for 1 hour, and then cryoprotected in a 30% sucrose/Tris-buffered saline solution (TBS; 0.2 M Tris, 0.15 M sodium chloride) overnight.

Immunohistochemistry

Cryoprotected brains were cut into three sets of 30-μm-thick coronal sections from the level of the medial septum through to the periaqueductal gray nucleus and underwent free-floating immunohistochemistry.

Double-label immunofluorescent labeling for green fluorescent protein (GFP) and kisspeptin was performed in one set of tissue to confirm the specificity of EGFPf tracer expression in kisspeptin neurons and to investigate the relationship between kisspeptin neurons in different brain regions. Brain sections were labeled serially with antibodies against kisspeptin and GFP. Although EGFPf expression could be clearly visualized without antibody labeling, conjugation to a GFP antibody and fluorophore enhanced the brightness and maintained the stability of the signal through confocal imaging. Kisspeptin was identified using

a polyclonal rabbit anti-kisspeptin-10 antibody (AC566; Dr Alain Caraty, Universite Francois Rabelais de Tours, Tours, France; 1:5000) (37), and EGFPf was labeled with a polyclonal chicken anti-GFP antibody (GFP-1020; Aves Labs Inc; 1:5000) (Table 1). Antibodies were diluted in TBS containing 0.3% Triton X-100, 0.25% bovine serum, albumin, and 2% normal goat serum (pH 7.6), and brain sections were incubated in this solution on an orbital shaker for 48 hours at 4°C. Sections were subsequently washed in TBS and incubated in secondary antibodies for 2 hours at room temperature. Secondary antibodies included Alexa fluorochromes A-11011 (Molecular Probes; 1:200) to visualize kisspeptin, and A-11039 (Molecular Probes; 1:200) to recognize GFP labeling. The sections were then washed, mounted onto gelatin-coated glass slide, air dried, and coverslipped with Fluoromount G (Southern Biotech).

Double-label immunofluorescent labeling for GFP and GnRH was performed in a second set of tissue to investigate contacts from distinct ARN or RP3V kisspeptin neurons to GnRH neurons. Brain sections were incubated in a cocktail of primary antibodies against GFP (GFP-1020; Aves Labs Inc; 1:5000) and GnRH (polyclonal guinea pig anti-GnRH; GnRH-GA2; Associate Professor Greg M. Anderson, University of Otago, Dunedin, New Zealand; 1:5000) (Table 1). Sections were incubated for 48 hours at 4°C as described above followed by detection with the following secondary antibodies: Alexa fluorochromes A-11039 and A-11075 (Molecular Probes; 1:200). Sections were mounted and coverslipped as described above. Omission of primary antibodies served as a negative control.

Microscopy and image analysis

The locations of ARN and RP3V injection sites were investigated in every third section using an epifluorescent microscope, Olympus BX-51 (Olympus Optical). Only animals with accurate and restricted injection sites were included in the analysis (ARN injections: n = 6 females, n = 5 males; RP3V injections: n = 4 females and n = 4 males). Coexpression of EGFPf and kisspeptin was quantified in two representative sections of the anteroventral periventricular nucleus (AVPV). The number of cells expressing EGFPf was quantified in eight representative sections through the rostral to caudal ARN.

The percentage of GnRH neurons contacted by EGFPf-positive kisspeptin fibers was quantified in two representative coronal sections at the level of the medial septum (MS), the rostral preoptic area (rPOA), and the anterior hypothalamic area (AHA). Sections were initially viewed under epifluorescence, and putative close appositions were confirmed with confocal microscopy. Confocal images were collected using a Zeiss LSM710 (Carl Zeiss) upright confocal microscope with an argon laser exciting at 488 nm and a helium laser exciting at 543 nm. Optical z-stack images for analysis of membrane close appositions between positive labels were acquired at 0.5- to 1.0-μm intervals with a Plan Neofluar ×40 objective with ×3 zoom function applied. Close apposition was defined by the absence of black pixels between green and red signals on a single z-stack plane. Maximum intensity projections of stacks and other image analyses were performed using LSM software Zeiss ZEN2009 and are presented as collapsed z-stacks of 15–25 optical images unless otherwise noted in the figure. Montages of projected images and adjustments of brightness levels were made in Adobe Photoshop CS5 and camera lucida-like reconstructions were manually drawn using Adobe Photoshop CS5.

Statistical analysis

Statistical analysis was performed using PRISM software (GraphPad). Male and female groups were compared using nonparametric Mann-Whitney *U* tests. In all statistical measures, a value of $P < .05$ was accepted as statistically significant.

Results

Targeting EGFPf expression to specific kisspeptin populations

Single unilateral injections of AdV-EGFPf into the AVPV in males and females resulted in EGFPf-expressing cells that were contained within the RP3V (AVPV and rostral to caudal extent of the periventricular nucleus) (Figure 1, A). A significantly greater number of transfected kisspeptin neurons was observed in the female (n = 4) compared with the male (n = 4) (31.8 ± 3.5 vs 14.5 ± 3.7 cells per section, respectively; $P < .05$) (Figure 1). Dual labeling for GFP and kisspeptin in the AVPV (Figure 1, B–G) revealed that 64.8% ± 8.8% and 49.9% ± 14.8% of EGFPf-positive neurons were colocalized with kisspeptin immunoreactivity in female and male mice, respectively. Although EGFPf-positive neurons were restricted to the zone of kisspeptin neurons in the RP3V, approximately 40% of EGFPf-positive neurons failed to label for kisspeptin peptide (Figure 1, F). Approximately half of the kisspeptin-immunoreactive neurons throughout the RP3V were targeted with EGFPf expression (52.8% ± 14.6% in females and 43.7% ± 10.7% in males).

Single unilateral injections of AdV-EGFPf into the ARN resulted in EGFPf-expressing cells contained specifically within the ARN (Figure 2, A) and extending throughout the rostral to caudal extent of the ARN (Figure

Table 1. Antibody Table

Peptide/Protein Target	Antigen Sequence (if Known)	Name of Antibody	Manufacturer, Catalog Number, and/or Name of Individual Providing the Antibody	Species Raised (Monoclonal or Polyclonal)	Dilution Used
GnRH	HWSYGLRPGGKRNTEHL	GA02	Associate Professor Greg M. Anderson	Guinea pig; polyclonal	1:5000
Kisspeptin-10	YNWNSFGLRY	AC566	Dr Alain Caraty	Rabbit; polyclonal	1:5000
GFP	Unknown	GFP-1020	Aves Labs Inc	Chicken; polyclonal	1:5000

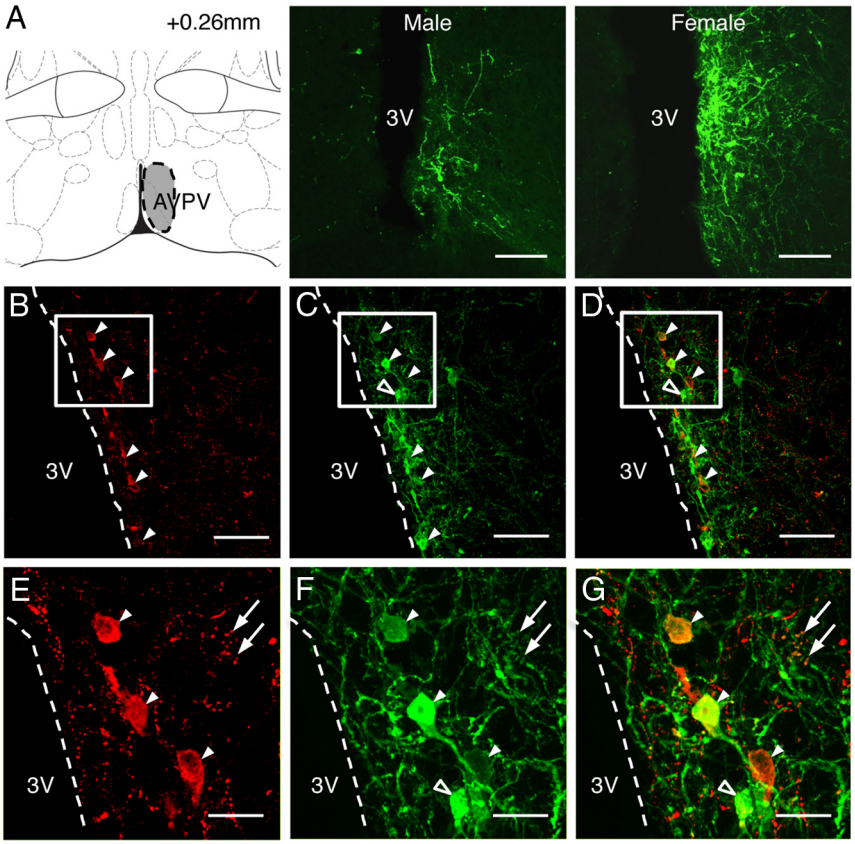

Figure 1. Targeting the RP3V kisspeptin population. A, Diagrammatic representation of a unilateral injection zone of EGFPf targeted to the AVPV of the Kiss-Cre mouse and representative confocal images of EGFPf-positive kisspeptin neurons within the AVPV of male and female mice. Scale bar, 200 μm. B–D, Representative confocal images showing double-labeled kisspeptin (red) and EGFPf tracer (green) in a female mouse. E–G, Enlarged images from boxed area in panels B–D. Colocalization of EGFPf and kisspeptin immunoreactivity in somata (arrowheads) and fibers (arrows). An EGFPf-positive neuron not immunoreactive for kisspeptin is indicated by open arrowhead. Scale bar, 100 μm (B–D) and 15 μm (E–G). 3V, third ventricle.

2, B). There were no significant differences in the total number of EGFPf-positive cells identified in males (n = 5) and females (n = 6) at any level of the ARN (87.0 ± 19.5 vs 126.3 ± 27.4 cells per section, respectively, across all eight sections). Very few EGFPf-positive cell bodies in the ARN were immunoreactive for kisspeptin, most likely due to the low level of peptide found in ARN kisspeptin cell bodies in gonadally intact animals (7)). However, transfected cells were restricted to the ARN and colocalization of GFP and kisspeptin could be observed in fiber processes. EGFPf was detectable throughout the soma, dendrites, and axons of transfected neurons in both regions. A small number of cells (one to three cells across all sections) positive for EGFPf was identified in the AVPV of some female mice after an injection into the ARN, suggesting a small degree of retrograde transport of the virus. Injection into the AVPV also yielded a small number of EGFPf-positive cells in the ARN (one to three cells across all sections). Injection of AdV-EGFPf into wild-type mice, lacking the Cre transgene, yielded no endogenous EGFPf expression in any region of the brain.

Kisspeptin projections to GnRH neurons in the rPOA from distinct subpopulations

Confocal imaging of GnRH neurons in two representative sections containing the MS, rPOA, and AHA was conducted to quantify close appositions from EGFPf-positive fiber processes originating in either the RP3V or the ARN. Mean GnRH neuron number investigated was 25.0 ± 1.7 in the MS, 46.5 ± 1.9 in the rPOA, and 8.1 ± 1.1 in the AHA across all the animals. In all male (n = 5) and female (n = 6) mice, the distribution of EGFPf-positive fibers coming from the ARN was notably sparse in the rPOA (Figure 3, A). A similarly sparse distribution of fibers was observed in the MS and AHA regions containing GnRH neurons. Nevertheless, some kisspeptin fibers originating from the ARN were observed near GnRH neurons and their proximal dendrites; however, no direct contacts were observed with confocal microscopy (Figure 3, Aa). In contrast, numerous kisspeptin fibers originating from RP3V neurons were observed surrounding GnRH neurons in the MS, rPOA, and AHA (Figure 3, B), and these fibers were frequently observed in close apposition with GnRH neuron cell bodies and proximal dendrites (Figure 3, Bb). GnRH neuron somata and proximal dendrites with either single or multiple contacts were counted from two representative sections through the MS, rPOA, and AHA of each mouse (Figure 3, C). Confocal imaging revealed a complete absence of contact between ARN kisspeptin fibers and GnRH neuron cell bodies or proximal dendrites in any region or either sex (Figure 3, D). RP3V kisspeptin fibers, however, were found to contact GnRH neurons throughout their distribution in females, contacting 3.8% ± 2.4% of MS GnRH neurons, 15.4% ± 2.3% of rPOA GnRH neurons, and 47.15% ± 6.6% of GnRH neurons in the AHA. Kisspeptin fibers traced out of the RP3V in males contacted fewer GnRH neurons, 0% in the MS, 6.7% ± 1.9% in the rPOA, and 19.0% ± 8.7% in the AHA. The percentage of GnRH neurons contacted by EGFPf-posi-

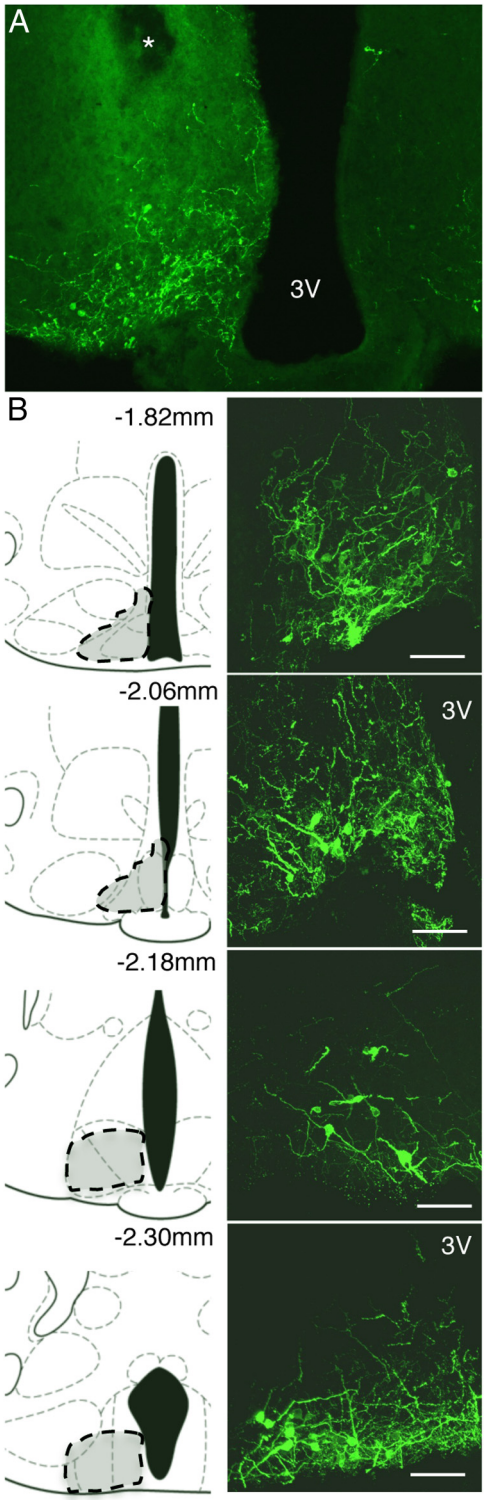

Figure 2. Targeting the ARN kisspeptin population. A, A representative epifluorescent image of EGFPf-expressing neurons in the ARN of a Kiss-Cre mouse injected with AdV-EGFPf. The asterisk indicates the placement of the syringe immediately ventral to the ARN. B, Representative schematic diagrams and corresponding projected confocal images showing the extent of EGFPf-positive neurons (shaded area) within the ARN of a representative female mouse. 3V, third ventricle. Scale bar, 100 μm (A) and 75 μm (B).

tive kisspeptin fibers from the RP3V was significantly higher in females compared with males in the rPOA and AHA (Figure 3, D).

Projections of ARN kisspeptin neurons throughout the brain

Although ARN kisspeptin fibers were not in contact with GnRH neuron cell bodies, they were identified in several preoptic nuclei (Figure 4, A and B), including the region of the organum vasculosum of lamina terminalis, AVPV, median preoptic nucleus, preoptic periventricular nucleus, medial preoptic nucleus, and medial and lateral regions of the septum. EGFPf-positive fiber processes originating from the ARN were also observed in the supraoptic nucleus, bed nuclei of stria terminalis, periventricular nucleus, AHA, paraventricular nucleus, lateral hypothalamic area (LHA), suprachiasmatic nucleus, posterior hypothalamic area, dorsomedial hypothalamic nucleus (DMH), throughout the ARN, medial part of the internal zone of the median eminence, lateral palisade zone (LPZ) of the external median eminence, supramammillary nucleus, and periaqueductal gray nucleus (Figure 4, A and B). It should be noted that in some animals with ARN injections, a small number of EGFPf-positive somata were identified in the LHA and DMH (Figure 4, B). However, when compared with animals in which EGFPf-positive somata were entirely restricted to the ARN, no notable differences in projection patterns were observed. Injection directly into the LHA in one animal resulted in EGFPf-positive somata restricted entirely to the LHA- and EGFPf-positive fibers also restricted to the LHA and ARN.

Projections between ARN kisspeptin neurons and AVPV kisspeptin neurons

EGFPf-positive fiber projections from the ARN kisspeptin neurons to the AVPV were present in both male and female mice; however, fiber processes appeared more abundant in female mice (Figure 5, A and B). In both male and female mice, EGFPf-positive fibers projecting from the ARN were found in close contact with kisspeptin cell bodies in the AVPV (Figure 5, Ai and Bii). Contacts from ARN kisspeptin fibers to AVPV kisspeptin neurons were observed in all of the female mice (n = 6) and in two of the five male mice investigated. Interconnectedness within the RP3V was also observed, with EGFPf-positive RP3V kisspeptin fibers in contact with immunoreactive kisspeptin neurons and vice versa (Figure 5, Ciii and iv).

The reciprocal fiber projection, from EGFPf-positive RP3V kisspeptin neurons to ARN kisspeptin neurons, was difficult to assess due to extremely low kisspeptin immu-

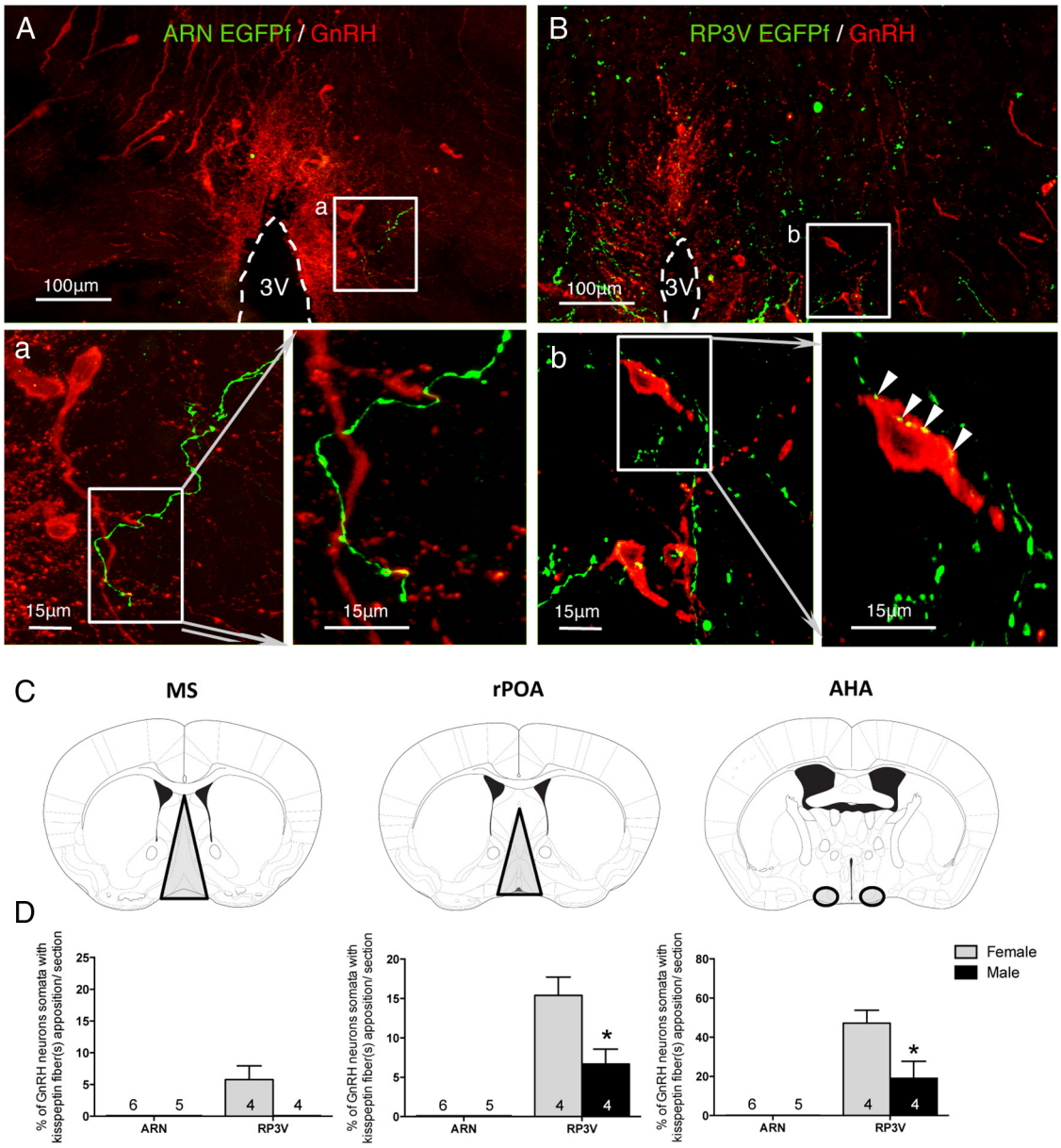

Figure 3. Kisspeptin contacts to GnRH neuron cell bodies and proximal dendrites from the distinct kisspeptin populations. A, Representative confocal image showing sparse EGFPf-positive ARN kisspeptin projections (green) within the region of GnRH neuron cell bodies (red) in the rPOA. An enlarged image of the region in panel a shows an ARN kisspeptin fiber near a GnRH neuron process in a projected confocal stack; however, no close apposition between the two was observed with confocal microscopy. B, Representative confocal images of dense EGFPf-positive RP3V kisspeptin fiber projections (green) within the region of GnRH neuron cell bodies (red) in the rPOA. The enlarged region in panel b shows close apposition between kisspeptin fibers originating in the RP3V (green) and GnRH neurons (arrowheads). C, Representative schematic diagrams of the coronal levels at which GnRH cell bodies were imaged for kisspeptin fiber contacts (shaded area). D, Percentage of GnRH neuron somata from each rostral to caudal area that received close contacts from kisspeptin fibers originating in the RP3V or ARN in male and female mice. *, $P < .05$ compared with female mice. 3V, third ventricle.

noreactivity in the cell bodies of ARN kisspeptin neurons. However, many EGFPf-positive RP3V kisspeptin fibers were located throughout the ARN when the RP3V population was targeted (Figure 6, C). Both in males and females, kisspeptin immunoreactive fibers were identified in close contact with EGFPf-positive ARN kisspeptin neurons; however, the origin of these fiber processes could be the RP3V or ARN (Figure 5, Dv).

Kisspeptin contacts to GnRH neuron projections within the median eminence

EGFPf-positive ARN kisspeptin fibers were found in close association with GnRH neuron projections at the pial surface of the base of the brain, the LPZ of the median eminence, and the medial part of the internal zone of median eminence, in which dense GnRH neuron processes were observed (Figure 6, A and B). Confocal microscopy

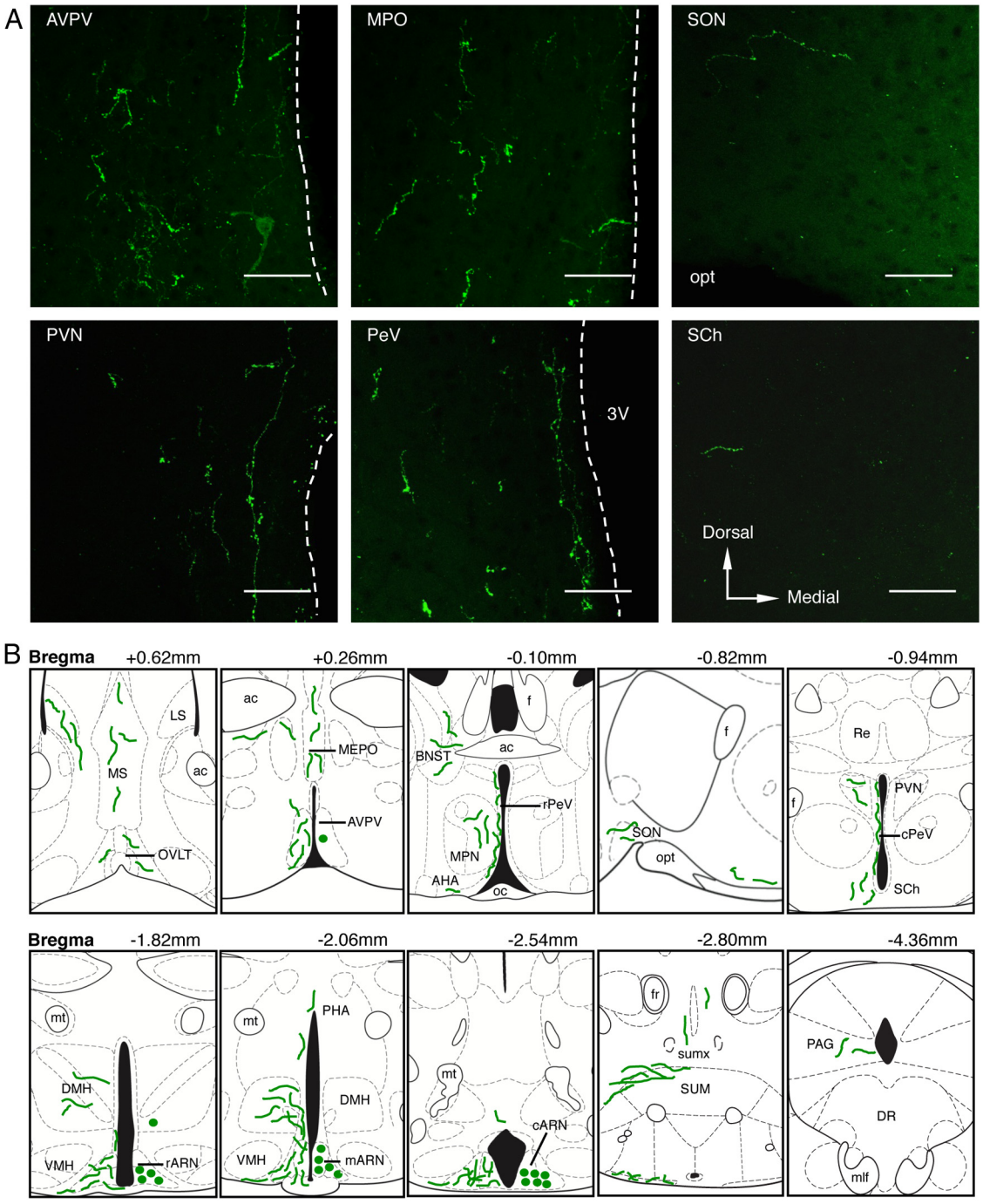

Figure 4. ARN kisspeptin fiber projections. A, Confocal images showing the distribution of EGFPf-positive ARN kisspeptin fibers throughout the AVPV, medial preoptic nucleus (MPO), supraoptic nucleus (SON), paraventricular nucleus (PVN), rostral and caudal periventricular nucleus (rPeV and cPeV), and suprachiasmatic nucleus (SCh) in a female mouse. B, Schematic diagrams of coronal brain sections arranged from rostral to caudal illustrating the distribution of EGFPf-positive ARN kisspeptin fibers (green lines) and somata (green dots) observed in a representative female mouse. ac, anterior commissure; BNST, bed nucleus of the stria terminalis; cARN, caudal ARN; DMH, dorsomedial hypothalamic nucleus; DR, dorsal raphe nucleus; f, fornix; fr, fasciculus retroflexus; LS, lateral septum; mARN, middle ARN; MEPO, median preoptic area; mlf, medial longitudinal fasciculus; MPN, medial preoptic nucleus; mt, mammillothalamic tract; oc, optic chiasm; opt, optic tract; OVLT, organum vasculosum of lamina terminalis; PAG, periaqueductal gray; PHA, posterior hypothalamic area; rARN, rostral ARN; Re, reuniens thalamic nucleus; SUM, supramammillary nucleus; sumx, supramammillary decussation; VMH, ventromedial hypothalamic nucleus. Scale bar, 60 μm.

confirmed, in both male and female mice, that EGFPf-positive fibers from the ARN were closely apposed to GnRH neuron processes at the pial surface of the base of the brain (Figure 6, A) as well as in the LPZ of the external median eminence and the medial part of the internal zone of median eminence (Figure 6, B). Contacts were evident

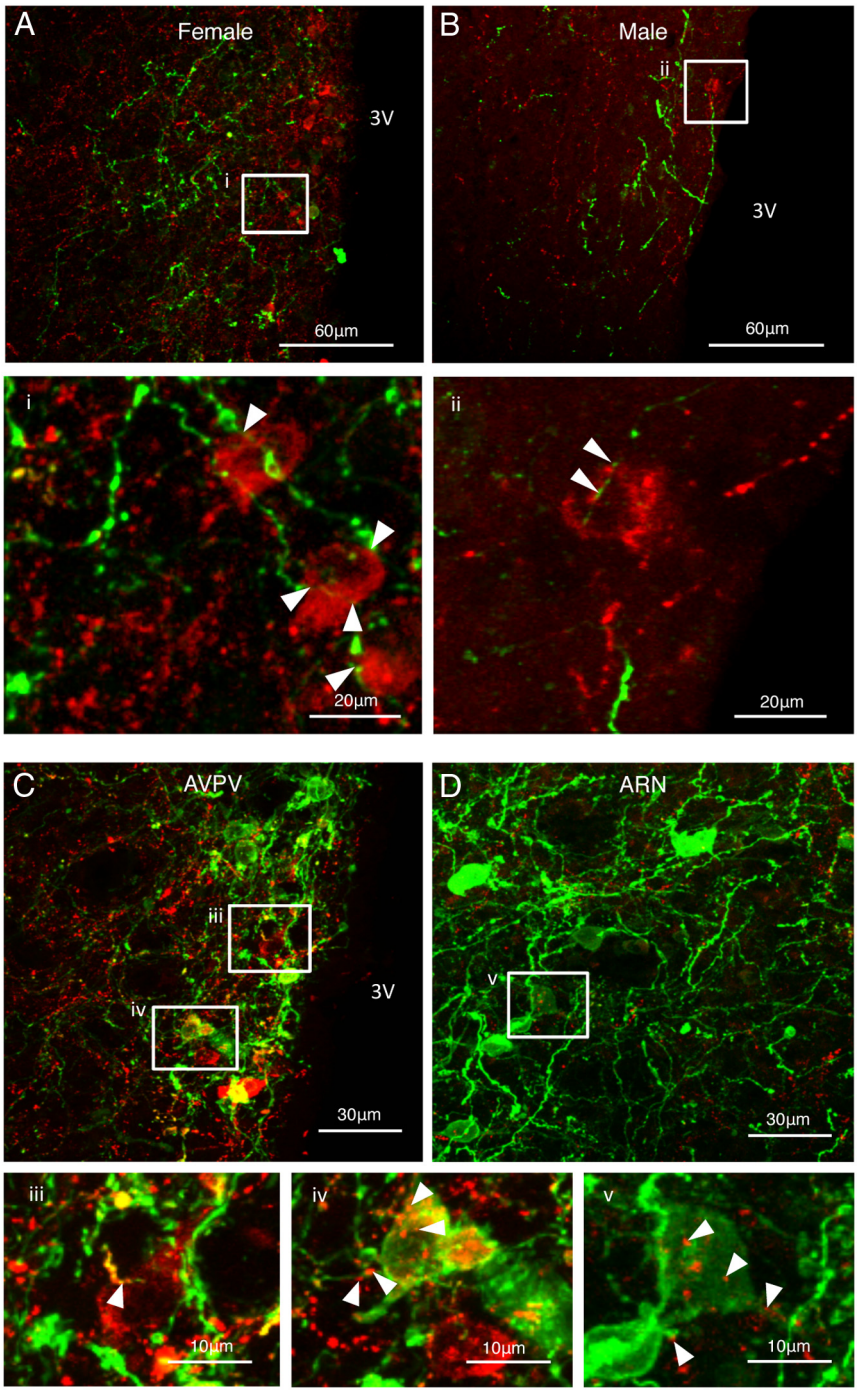

Figure 5. Kisspeptin to kisspeptin contacts. Confocal images of the AVPV in representative female (A) and male (B) mice showing EGFPf-positive ARN kisspeptin fibers (green) closely apposed to immunolabeled kisspeptin neurons. i and ii, Enlarged images from boxed areas in panels A and B. C, RP3V kisspeptin neurons filled with EGFPf (green) are surrounded by EGFPf-positive and -negative kisspeptin fibers (yellow and red). Enlarged images in panel iii show RP3V kisspeptin immunoreactive neuron (red) closely apposed by EGFPf and kisspeptin colabeled fibers (yellow) and panel iv shows RP3V kisspeptin neuron filled with EGFPf (green) closely apposed by kisspeptin immunoreactive fibers (red). D, ARN kisspeptin neurons filled with EGFPf (green) are closely apposed by kisspeptin immunoreactive fibers (red). Arrowheads indicate sites of close contacts. 3V, third ventricle.

between EGFPf-positive fibers and GnRH neuron fibers (Figure 6, Ai and B, ii, iii, and iv). Similarly, EGFPf-positive kisspeptin fibers projecting from the RP3V in both male and female mice were observed in all of the same regions (Figure 6, C), and close appositions were also identified between EGFPf-positive fibers and GnRH neuron projections in both male and female mice (Figure 6C, v, vi, and vii).

GnRH neuron projections to kisspeptin processes within the arcuate nucleus

Unexpectedly, the GnRH neuron projections were also observed in close contact with EGFPf-positive ARN kisspeptin dendrites and axons (Figure 7). In some cases, GnRH-positive fibers were found closely apposed to dendritic spines of EGFPf-positive ARN kisspeptin neurons (Figure 7, Ai). A montage of confocal z-stack projections of a single EGFPf-positive ARN kisspeptin neuron from a representative female mouse is shown in Figure 7, B and C, displaying a thick dendritic projection and a thin axonal projection (diameters of approximately 1.5 μm and 0.5 μm, respectively). Camera lucida-like reconstruction of this ARN kisspeptin neuron (Figure 7, B) revealed dendritic spines along the dendrite (Figure 7, Biii) and a frequently beaded axonal projection (Figure 7B, iv). Close interactions between the GnRH neuron projections and the EGFPf-positive ARN kisspeptin neuron were apparent at both the dendritic and axonal level (Figure 7A, i and ii, and 7B, iii and iv).

Discussion

The present study investigated the specific fiber projection patterns of the anatomically distinct kisspeptin populations in the RP3V and ARN of the mouse and determined their contact with GnRH neuron cell bodies, proximal dendrites, and distal processes (summarized in Figure 8). We found that kisspeptin contacts to GnRH neuron cell bodies and proximal dendrites in the rostral preoptic area originate entirely

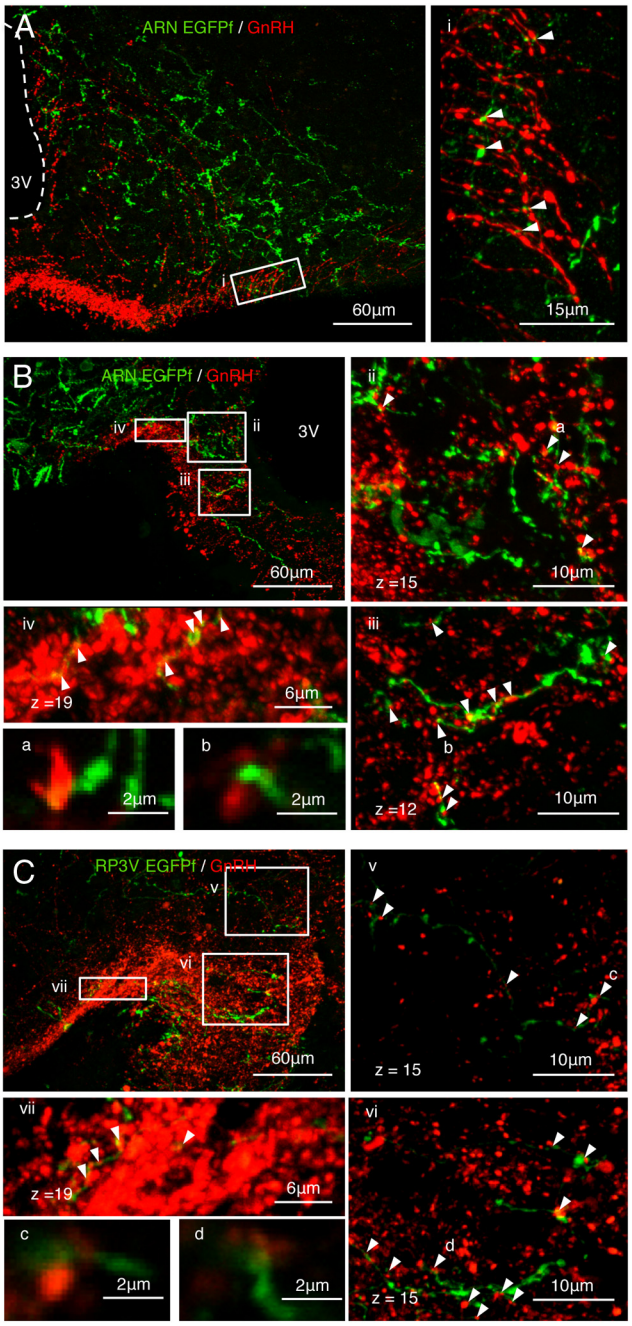

Figure 6. Kisspeptin contacts to GnRH neuron projections within the median eminence. Representative confocal images showing EGFPf-positive ARN kisspeptin fibers (green) closely apposed to GnRH neuron processes (red) near the pial surface of the base of the brain (A) and in the LPZ of the external median eminence and medial part of the internal zone of the median eminence (B) in a representative female mouse. C, Representative confocal images showing close apposition of EGFPf-positive RP3V kisspeptin fibers closely apposed to GnRH neuron processes (red) in the LPZ of the external median eminence and medial part of the internal zone of the median eminence of a female mouse. Panels i, ii–iv, and v–vii are high-magnification images from corresponding boxed areas in panels A, B and C. Panels a–d show single confocal slices (1 μm optical thickness) displaying examples of close apposition by the absence of black pixels between green and red signals. z refers to the number of confocal optical images in the z-plane acquired at 0.5 μm intervals. 3V, third ventricle.

from kisspeptin neurons located in the RP3V. Although fibers from ARN kisspeptin neurons were identified extending as far as the midbrain and within the rPOA, no direct contact with GnRH cell bodies or proximal dendrites was observed. In contrast, the distal processes of GnRH neurons in the ARN and median eminence were observed to have close appositions from kisspeptin fibers originating from both the RP3V and ARN. Of interest, ARN kisspeptin fibers were concentrated in the AVPV, to a greater extent in females, and the fibers of ARN kisspeptin neurons were found in direct contact with RP3V kisspeptin neurons, suggestive of communication between the two populations. Similarly, GnRH neuron processes were found in close contact with ARN kisspeptin neuron dendrites, suggesting a neuroanatomical basis for feedback between GnRH and kisspeptin neurons.

The advantage of the currently applied technical approach over previous tract-tracing and labeling studies was the ability to target the kisspeptin neurons exclusively, within each brain region independently, with a highly effective tracing molecule. In addition, this approach is not dependent on the detection of coexpressed peptides that are sensitive to the gonadal steroid hormone environment. A precise, small volume injection of a Cre-dependent transgene expressing a farnesylated EGFP (EGFPf) (35, 36) into each nucleus was able to largely restrict the expression of EGFPf to kisspeptin neurons within that nucleus. EGFPf docks in the membrane of neurons throughout their extent, resulting in neurons that resemble neurobiotin or biocytin filled neurons (38, 39). This enables visualization of the full morphology of the targeted neuron and the ability to follow fiber projections throughout their length. A very small number of cell bodies were found expressing EGFPf well outside the injection zone, most likely due to the uptake and retrograde transport of the viral vector from nerve terminals.

Previous characterization of the kiss-Cre mouse has reported faithful and specific Cre activity in the vast majority of kisspeptin neurons in the ARN of males and females and the RP3V of females (15, 34). In these brain regions, greater than 90% of Cre-expressing cells are found to express kisspeptin (34). In males, kisspeptin-Cre reporter expression is less specific within the RP3V, with approximately 60% of Cre reporter-labeled neurons coexpressing kisspeptin (34). In the present study, the identity of EGFPf-expressing neurons was confirmed with immunohistochemical colocalization within the cell bodies of the RP3V, in which the peptide expression can be readily detected in somata, and in fiber processes of the ARN. Approximately half of the kisspeptin neurons expressed EGFPf in the RP3V of males and females. A larger-than-expected proportion of EGFPf-only expressing cells were

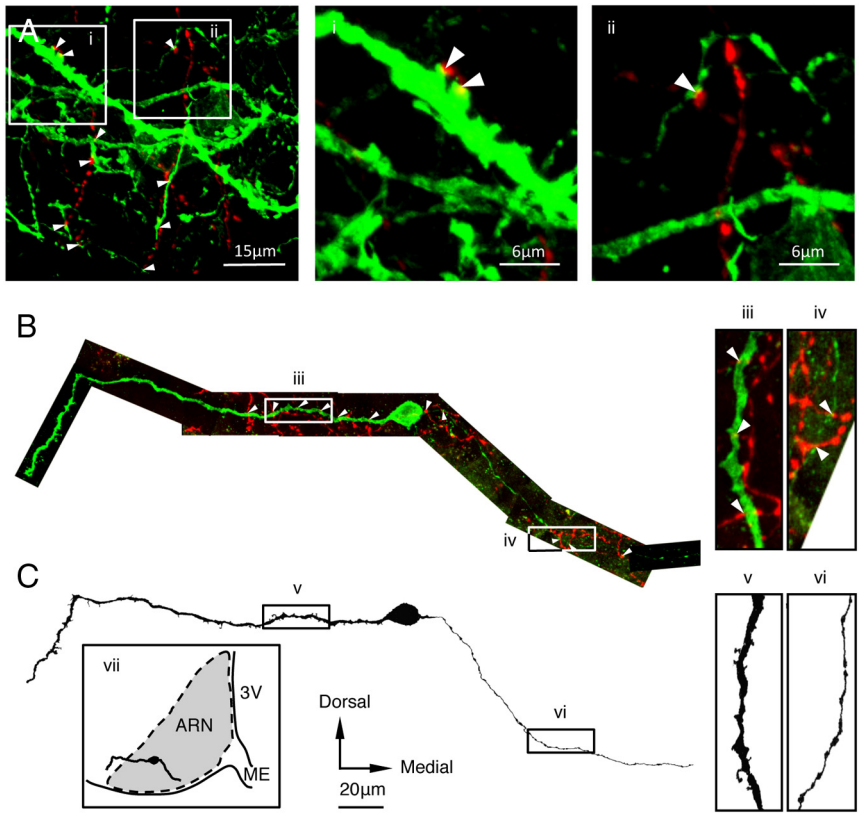

Figure 7. GnRH neuron projections to ARN kisspeptin neurons. A, Representative confocal image showing GnRH fiber processes (red) closely apposed to EGFPf-positive kisspeptin fibers (green) in the ARN. i–ii, Enlarged view of boxed area in panel A. B, Montage of confocal z-projections of an EGFPf-positive ARN kisspeptin neuron. C, A camera lucida-like reconstruction exhibiting a typical thick dendrite decorated with multiple spines (iii and v) and a thin axon with boutons (iv and vi). vii, Schematic diagram showing the position of the EGFPf-positive ARN kisspeptin neuron. Arrowheads indicate close apposition of GnRH fibers (red) and EGFPf-positive kisspeptin dendrites and axons (green). 3V, third ventricle.

identified within the RP3V, given the specificity of the Cre activity reported previously for the Kiss-Cre mouse (34). This could be due to ectopic or developmental Cre expression in adult neurons that no longer express kisspeptin as an adult or an inability to detect the kisspeptin peptide in these neurons due to low expression or an impaired ability to immunohistochemically label peptide in cells that have EGFPf docked throughout the membrane. Using a similar approach in GnRH-Cre animals, we have found that EGFPf expression reduces our ability to detect the GnRH peptide in cells that are otherwise morphologically and anatomically characteristic of GnRH neurons (40). Therefore, the lack of kisspeptin labeling in some transfected cells may be due to a technical limitation as opposed to identifying ectopic expression of the tracer in non-kisspeptin neurons. In support of this possibility, no EGFPf expression was present in any brain region of wild-type-injected mice.

Our results provide support for and also extend previous work investigating kisspeptin neuroanatomical circuits in the rodent. Anterograde and retrograde tracing studies in adult female mice (27) and rats (29) report that the projection patterns of kisspeptin neurons are diverse and somewhat distinct, with ARN kisspeptin neurons projecting medially and laterally and to limbic structures, and RP3V kisspeptin neurons projecting predominantly medially. We identified fibers from the ARN projecting to many of the same regions reported previously; however, there were some notable differences. The present study identified ARN kisspeptin fibers in the lateral septum and AHA, areas not identified with *Phaseolus vulgaris* Agglutinin, PHA-L anterograde tracing coupled with IHC for kisspeptin (27), suggesting that the present technique was able to sample a larger proportion of the ARN kisspeptin population than could be targeted with discreet PHA-L injections. However, despite targeting a large number cells throughout the rostral to caudal extent of the ARN, kisspeptin fibers were not identified in close contact with any GnRH neuron cell bodies or proximal dendrites in regions from the MS to the AHA. Sparse ARN kisspeptin fibers were observed within the rPOA in the present study, similar to other studies in which the coexpression of neurokinin B was used to identify ARN kisspeptin fibers in the rat (29, 41). Previous studies report the uptake of fluorogold in approximately 20% of the rostral ARN kisspeptin neurons after injections into the rPOA (28); however, the only direct evidence for ARN kisspeptin synapses onto GnRH cell bodies comes from a study by Kallo et al (31), who reported that a very small proportion, approximately 5%, of kisspeptin synapses to GnRH neuron somata coexpress neurokinin B.

Our data support the notion that the vast majority of the direct kisspeptin input to GnRH neurons in the region of their cell bodies and proximal dendrites comes from the RP3V kisspeptin neurons in the mouse. However, both populations appear to be in direct contact with GnRH processes and terminals within the ARN and median eminence. Evidence to support synaptic contact from ARN kisspeptin neurons to GnRH neuron processes comes from genetic transsynaptic tracing of GnRH afferent inputs during development in the mouse (42). Previous re-

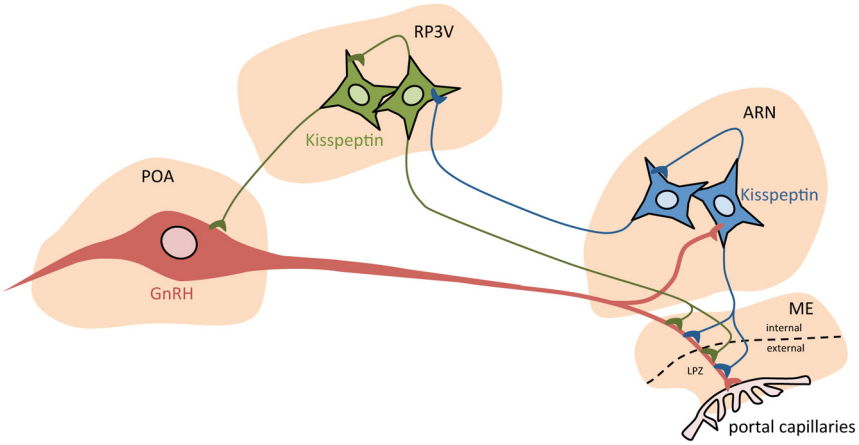

Figure 8. Diagram illustrating the interactions between RP3V and ARN kisspeptin neurons and GnRH neurons. Viral-mediated tracing of fiber projections from the distinct kisspeptin populations demonstrates selective areas of contact to GnRH neuron somata and fiber processes and interconnectedness between the distinct kisspeptin populations and GnRH neurons. RP3V kisspeptin neurons (green) project heavily to GnRH neuron somata and proximal dendrites in the preoptic area (POA) and to GnRH neuron distal processes in the ARN, internal median eminence (ME), and lateral palisade zone (LPZ) of the external median eminence. ARN kisspeptin neurons (blue) project to the RP3V and contact RP3V kisspeptin neurons but do not contact GnRH neuron somata or processes in the POA. ARN kisspeptin neurons contact distal GnRH neuron processes in the ARN, internal median eminence, and lateral LPZ. GnRH neurons reciprocally contact ARN kisspeptin neurons and interconnectedness between kisspeptin neurons is evidence in both the RP3V and ARN.

ports in the mouse (27) and rat (25, 41, 43) have reported limited or absent kisspeptin fibers in the external zone of the median eminence, suggesting that kisspeptin acts through either volume transmission to GnRH nerve terminals or through dendronic modulation preceding the GnRH nerve terminal (44). Our data show that most kisspeptin fibers from both populations are found in the internal zone of the median eminence; however, fibers are also identified in the LPZ of the external median eminence in which most GnRH neuron fibers are found. Fluorogold experiments suggest that ARN kisspeptin neurons are not hypophysiotropic (27), but do not rule out the possibility that kisspeptin fibers directly regulate GnRH nerve terminals in the LPZ. The physiological relevance of kisspeptin input to distinct portions of the GnRH neuron is not entirely clear; however, these neuroanatomical findings provide clues as to how kisspeptin might evoke different patterns of GnRH activity and release. For example, kisspeptin signaling from RP3V neurons to the GnRH cell body and proximal dendrite are most likely important for modulating the firing pattern of the cells and potentially mediating signaling cascades that lead to expression of other signaling molecules. Kisspeptin actions near GnRH nerve terminals, probably originating from both populations of cells, may be more important in regulating peptide hormone release. Recent work describes region-specific regulation of GnRH secretion by kisspeptin in the POA and median eminence (45) that may facilitate the different functions of kisspeptin signaling from their distinct populations. It remains to be determined whether RP3V kisspeptin neurons send collaterals to both the GnRH neuron somata and terminal region or whether these inputs arise from separate populations of cells.

The specific roles of the distinct kisspeptin subpopulations may differ between different mammalian species (46). In sheep, GnRH somata receive direct innervation from kisspeptin neurons residing in both the POA and ARN (12). Subpopulations of ARC kisspeptin neurons in the ewe are activated during both surge and pulsatile modes of secretion (47), suggesting that unlike in the rodent, in which the RP3V kisspeptin population is considered central for estradiol positive feedback, populations of ARN kisspeptin neurons in the ewe might play a role in mediating both positive and negative feedback actions of estradiol on GnRH secretion. In humans, kisspeptin neurons in the infundibular region are suggested to be the major source of direct kisspeptin innervation to GnRH neuron cell bodies (5, 48); however, distinct markers of the preoptic area and infundibular kisspeptin populations are required to fully address this possibility.

Similar to what has been reported previously in classical tract-tracing studies (27), we observed evidence for reciprocal connections between the ARN and RP3V neurons. Dense fiber projections from filled ARN kisspeptin neurons were observed in the AVPV and in close contact with kisspeptin somata. These data suggest that, in addition to the well-described intra-ARN connections between kisspeptin neurons (12, 29, 32), ARN kisspeptin neurons also connect with RP3V kisspeptin neurons. As found in the ARN, we also observed anatomical evidence for intra-RP3V kisspeptin connections. The evidence for RP3V kisspeptin innervation of ARN kisspeptin neurons is less clear due to limitations in visualizing ARN kisspeptin cell bodies. However, an AVPV to ARN pathway is well characterized in the rodent (48), and multiple cell types in the ARN are known to be regulated by kisspeptin, including neurons that express dopamine (49), neuropeptide Y (50), and proopiomelanocortin (51). Together these neuroanatomical links suggest that reciprocal signaling that may play an important role in the feedback regulation of global kisspeptin signaling to GnRH neurons in the regulation of

fertility. Curiously, ARN and RP3V kisspeptin neurons do not respond electrically to kisspeptin (34, 52), suggesting that other coreleased neurotransmitters are involved.

GnRH nerve terminals identified in close contact with dendritic and axonal elements of EGFPf-filled kisspeptin neurons in the ARN suggests that additional feedback regulation may exist from GnRH neurons to kisspeptin neurons in the ARN. Previous reports have identified GnRH contacts to kisspeptin neuron cell bodies in the medial basal hypothalamus of sheep (6) and to approximately 50% of the ARN kisspeptin neuron cell bodies in mice, in which synaptic inputs were confirmed with electron microscopy (53). In the present study, we have identified anatomical evidence for close contact between GnRH axonal elements and the dendritic spines of ARN kisspeptin neurons, suggesting that GnRH, or potentially glutamate released from GnRH nerve terminals, may be acting at kisspeptin neurons via dendritic spines. This additional reciprocal innervation may provide critical feedback or phasic information required for pulse generation.

In summary, using a viral-mediated, tract-tracing approach, we have selectively mapped out the projections of RP3V and ARN kisspeptin neurons. Our data suggest these anatomically distinct populations have differential roles in modulating GnRH neuron function and release while also possessing a high degree of reciprocal feedback communication between the two populations and GnRH neurons. Together the results of these studies contribute to our knowledge of the neuroanatomical framework underpinning the physiological regulation of GnRH neurons and fertility by kisspeptin neurons in the RP3V and ARN.

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