Projections of Arcuate Nucleus and Rostral Periventricular Kisspeptin Neurons in the Adult Female Mouse Brain

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The important role of kisspeptin neurons in the regulation of GnRH neuron activity is now well accepted. However, the ways in which kisspeptin neurons located in the arcuate nucleus (ARN) and rostral periventricular area of the third ventricle (RP3V) control GnRH neurons are poorly understood. The present study used anterograde and retrograde tracing techniques to establish the neuronal projection patterns of kisspeptin cell populations in the female mouse brain. Anterograde tracing studies revealed that kisspeptin neurons in the ARN innervated a wide number of hypothalamic and associated limbic region nuclei, whereas RP3V kisspeptin neurons projected to a smaller number of mostly medially located hypothalamic nuclei. Retrograde tracing confirmed a major projection of RP3V kisspeptin neurons to the ARN and showed that kisspeptin neurons located in the rostral half of the ARN projected to the rostral preoptic area. Peripheral administration of Fluorogold was found to label the majority of GnRH neurons but no kisspeptin neurons. Together, these studies highlight the complexity of the brain kisspeptin neuronal system and indicate that both ARN and RP3V kisspeptin neurons participate in a variety of limbic functions. In relation to the GnRH neuronal network, these investigations demonstrate that, alongside the RP3V kisspeptin cells, rostral ARN kisspeptin neurons may also project to GnRH neuron cell bodies. However, no kisspeptin neurons innervate GnRH nerve terminals in the external layer of the median eminence. These studies provide a neuroanatomical framework for the further elucidation of the functions of the ARN and RP3V kisspeptin neuron populations. **(*Endocrinology* 152: 2387–2399, 2011)**

Kisspeptin neurons are now established to have key roles in the regulation of the hypothalamo-pituitary-gonadal axis. In all mammalian species examined to date, two major populations of kisspeptin neurons have been identified, one located in the arcuate nucleus (ARN) and another in the preoptic area (1, 2). It is thought that these two populations have different physiological roles in the control of gonadal function. Experiments indicate that the preoptic kisspeptin neurons, located within the rostral periventricular area of the third ventricle (RP3V) of the mouse (3), have key roles in the activation of GnRH neurons to evoke the preovulatory GnRH/LH surge as well as pubertal maturation in females (4–6). In contrast, it has been proposed that the arcuate kisspeptin neurons contribute to estrogen negative feedback mechanisms and, possibly, GnRH neuron pulsatility in rodents, sheep, and goats (5, 7–9).

Precisely how the different kisspeptin populations control the activity of GnRH neuron is not known. Electrophysiological and imaging data have demonstrated that kisspeptin acts at the level of the GnRH neuron cell body and dendrite (10–13), whereas *in vitro* evidence has raised

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Abbreviations: AP, Anterior-posterior; ARN, arcuate nucleus; AVPV, anteroventral periventricular nucleus; BBB, blood brain barrier; BDA, biotinylated dextran amine; BST, bed nuclei of the stria terminalis; cARN, caudal ARN; DMN, dorsomedial nucleus; FG, Fluorogold; LHA, lateral hypothalamic area; LS, lateral septum; ME, median eminence; MEPO, median preoptic nucleus; MPN, medial preoptic nucleus; OVX, ovariectomized; PAG, periaqueductal gray; PHA-L, *Phaseolus vulgaris* Agglutinin; PV, paraventricular thalamic nucleus; PVN, paraventricular nucleus; PVpo, preoptic periventricular nucleus; rPOA, rostral preoptic area; rARN, rostral ARN; RP3V, rostral periventricular area of the third ventricle; TBS, Tris-buffered saline; VMN, ventromedial nucleus.

the possibility that kisspeptin may also regulate GnRH neuron nerve terminals in the median eminence (ME) (14, 15). On the basis of correlative data, we have previously made the case that the RP3V kisspeptin neuron population targets the GnRH neuron cell body/dendrite (11), and it has been assumed that the arcuate kisspeptin neurons target the GnRH nerve terminals (1). However, definitive studies are lacking, and it remains that the projections of arcuate and RP3V kisspeptin neurons are not known. The importance of understanding the innervation patterns of the different kisspeptin neuron populations is further emphasized by the growing awareness that the hypothalamic kisspeptin neurons innervate a wide variety of neurons (16, 17), some of which appear to be outside the GnRH neuronal network.

In the present study, we have used retrograde and anterograde tract-tracing approaches to provide detailed information on the projections of both the RP3V and arcuate kisspeptin neuron populations in the female mouse.

Materials and Methods

Animals

Adult (age 2–3 months old) female C57BL/6J mice were housed under a 12-h light, 12-h dark cycle (lights on 0600 h) with *ad libitum* access to food and water. All animal experimental procedures were undertaken with the authorization of the University of Otago Animal Welfare and Ethics Committee.

Antibodies

Both sheep and rabbit polyclonal antimouse kisspeptin-10 antibodies were provided by Alain Caraty (Institut National de la Recherche Agronomique, Tours, France). The rabbit polyclonal antibody raised against mouse kisspeptin-10 (AC566) has been well characterized (3, 18). The sheep polyclonal kisspeptin-10 antibody (AC024) was generated by coupling mouse kisspeptin-10 (YNWNSFGLRY-NH2) to bovine thyroglobulin and using it as an immunogen in sheep according to previously reported procedures (19). Specificity experiments consisted of overnight adsorption of AC024 with 1 μM kisspeptin-10 peptide and immunocytochemistry on brain sections from *Kiss1* knockout mouse (20). Antibodies directed against GnRH (polyclonal sheep; gift of Christine Molter-Gérard; Institut National de la Recherche Agronomique), *Phaseolus vulgaris* Agglutinin (PHA-L) (goat polyclonal; Vector Labs, Burlingame, CA), and Fluorogold (FG) (polyclonal rabbit; Millipore, Temecula, CA) have all been characterized previously (21–25).

Experiment 1. Anterograde tracing of kisspeptin neuronal projections

A total of 16 intact female mice (2–3 months old of age; weight, 19–25 g) were used for unilateral injections of PHA-L into the ARN. Mice were anesthetized with Halothane, placed in stereotaxic frame, and a glass microelectrode (outer diameter, 1.5 mm; tip diameter, 15–20 μm) containing 2.5% PHA-L in 0.1 M PBS (pH 8) was delivered iontophoretically with positive current (5 μA, 7 sec on, 7 sec off) for 18 min into the ARN [anterior-posterior (AP), 1.4–1.6 mm; lateral, 0.1 mm; depth, 6.4 mm; according to the atlas of Paxinos and Franklin (26)]. Unilateral anterograde tracer injections into the anteroventral periventricular nucleus (AVPV) and preoptic periventricular nucleus (PVpo), jointly termed the RP3V, were undertaken using biotinylated dextran amine (BDA) (Molecular Probes, Carlsbad, CA). The tip of a Hamilton syringe was positioned in the RP3V [AP, 0.6 mm; lateral, 0.2 mm; depth, 5.3 mm; according to the Paxinos and Franklin atlas (26)] and 250 nl of 2.5% BDA in PBS injected over 15 min with the syringe left *in situ* for 10 min before withdrawal. Five to 6 d after PHA-L/BDA delivery, animals were anesthetized with pentobarbital (3 mg/100 μl, ip) and transcardially perfused with 15 ml of 4% paraformaldehyde in PBS followed by post-fixation in the same fixative at room temperature for 1 h. The brains were placed in 30% sucrose/Tris-buffered saline (TBS) [0.5 M Tris and 0.15 M sodium chloride (pH 7.6)] at 4 C overnight before three sets of 30-μm-thick coronal sections were cut from the level of medial septum through to the midbrain.

Free-floating, dual-immunofluorescence labeling was undertaken to visualize uptake of PHA-L or BDA in kisspeptin-immunoreactive cells and fibers on one of the 1:3 sets of coronal sections. The PHA-L antiserum (1:1000) was combined with AC566 (1:4000) in TBS as a primary antibody cocktail for brains injected with PHA-L, whereas only AC566 was used with sections from BDA injections. Sections were incubated for 48 h at 4 C with the primary antibody cocktail in 2% normal horse or donkey serum in TBS containing 0.3% Triton-X-100 and 0.25% BSA. PHA-L sections were then washed and placed in biotinylated horse antigoat secondary immunoglobulins (1:200; Jackson ImmunoResearch, West Grove, PA) for 90 min at room temperature. Both PHA-L and BDA sections were then incubated in fluorescein isothiocyanate-labeled donkey antirabbit immunoglobulins (1:200; Jackson ImmunoResearch) and rhodamine avidin (1:200; Vector Labs). Sections were then washed, mounted on slides, air dried, and cover slipped with Vectashield Fluorescence Mounting Medium (Vector Labs). Controls consisted of the omission of primary and/or secondary antibodies for the different combinations, and these sections consistently failed to exhibit the appropriate immunofluorescence.

Analysis

The site and size of tracer injections was established by taking photomicrographs of every third coronal section rostral and caudal to the center of the injection site and drawing the extent of tracer deposition onto schematic diagrams form the Paxinos and Franklin Mouse Atlas (26). Boundaries for the injection site were defined as the area containing densely stained PHA-L/BDA soma with dendrites. Representative examples of the full extent of the PHA-L and BDA injection sites in individual mice are given in Supplemental Figs. 1 and 2, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org. In addition, the number of dual-labeled kisspeptin+PHA-L or BDA cell bodies was determined in three coronal brain sections through the center of the injection site in each animal. Brain sections from each mouse were then examined for the presence of dual-labeled PHA-L/BDA+kisspeptin fibers with ×60 or ×100 objectives and their locations mapped on to schematic brain maps. Stacked images were captured at 1-μm increments along the z-axis of each section with ×63 plan apochromat objective.

Experiment 2. Retrograde tracing of ARN kisspeptin projections to the rostral preoptic area (rPOA)

Eight mice (2–3 months of age, 19–25 g) were ovariectomized (OVX) under halothane anesthesia. OVX mice were used so as to increase the number of kisspeptin-immunoreactive cells visible in the ARN (27). A week after OVX, mice were anesthetized with Avertin (0.1 ml/3 g body weight) and placed in a stereotaxic frame. The tip of a Hamilton syringe was lowered into the rPOA (AP, 1.0 mm; lateral, 0.01 mm; depth, 5.2 mm) and 50 nl of 2% FG (Biotium, Hayward, CA) in sterile 0.9% saline injected over a period of 2 min with the syringe left in place for 5 min after injection before withdrawal of the needle. Animals were allowed to recover and 4 d later reanesthetized and perfusion fixed. Coronal sections were processed for immunocytochemistry as detailed above but using a primary antibody cocktail of sheep kisspeptin AC024 (1:2000) and rabbit anti-FG (1:5000). To determine the spatial relationship of FG deposition in relation to GnRH neuron cell bodies, rPOA sections were incubated in sheep GnRH (1:1000) and rabbit anti-FG (1:5000) antibodies. After 48 h at 4 C in the primary antibodies, sections were washed and placed in biotinylated donkey antisheep immunoglobulins (1:200; Jackson ImmunoResearch) for 90 min at room temperature. Sections were then washed and incubated with tetramethyl rhodamine isothiocyanate-conjugated donkey antirabbit immunoglobulins (1:200; Jackson ImmunoResearch) and fluorescein avidin (1:200; Vector Labs). The sections were mounted on slides, air dried, and cover slipped with mounting medium (Vector Labs).

The locations of FG deposition were determined by examining rPOA brains sections under the UV fluorescence. Evaluation of the FG injection sites was the same as that used for PHA-L/BDA injection sites. Supplemental Fig. 3 illustrates the extent of a FG injection site in one animal alongside the distribution of GnRH neurons within the area of injection. Brain sections containing the rostral ARN (rARN) (plates 45–47) and caudal ARN (cARN) (plates 48–50 of Paxinos and Franklin) were examined in each animal and the numbers of single-labeled kisspeptin and dual-labeled FG+kisspeptin neurons determined in two sections at each level. The values were averaged and used to generate group means ± SEM.

Experiment 3. Retrograde tracing of RP3V kisspeptin projections to the ARN

Thirteen intact mice (2–3 months of age, 19–25 g) were anesthetized with Avertin (0.1 ml/3 g body weight) and placed in a stereotaxic frame. The tip of a Hamilton syringe was lowered into the right ARN (AP, 1.6 mm; lateral, 0.3 mm; depth, 6.2 mm) and 50 nl of 2% FG injected over a period of 2 min as detailed above. Animals were allowed to recover and 4 d later reanesthetized, perfusion fixed, and coronal brain sections prepared and reacted for FG and kisspeptin immunofluorescence as detailed in experiment 2. A representative example showing the full extent of FG deposition in the ARN is shown in Supplemental Fig. 3. Analysis was the same as that detailed in experiment 2 with the exception that RP3V sections [AVPV (plate 28–29) and PVpo (plate 30–32)] were analyzed for FG+kisspeptin neurons.

Experiment 4. Retrograde tracing of kisspeptin neuronal projections into regions outside the blood brain barrier (BBB)

Mice (2–3 months of age, 19–25 g, n = 5/group) were OVX or sham operated under halothane anesthesia. A week after surgery, all animals were given an ip injection of 0.5 mg FG in 100 μl sterile saline. Animals were allowed to recover and 6 d later reanesthetized, perfusion fixed, and coronal brain sections processed for FG and kisspeptin immunofluorescence as detailed in experiment 2. Analysis was the same as that detailed in experiment 2 with the exception that both RP3V and ARN sections were analyzed for FG+kisspeptin neurons. In addition, two rPOA sections from each animal were examined for the numbers of GnRH neurons and dual-labeled FG+GnRH neurons. Average values were obtained from all animals and used to generate group means ± SEM. Statistical analysis was undertaken with Student's *t* test to compare the number of 1) kisspeptin neurons in AVPV, PVpo, and ARN; and 2) GnRH neurons in OVX and sham-operated animals.

Results

Experiment 1. Anterograde tracing of kisspeptin neuronal projections

ARN

Discrete PHA-L injections within the ARN (diameter, 200–300 μm) (Fig. 1, A and B, and Supplemental Fig. 1) were obtained in six of the 16 animals. The anterograde tracer was deposited into either the rARN (n = 4) or cARN (n = 2). Examination of these ARN sections showed that 3 ± 0.2 kisspeptin cells were labeled with PHA-L per three sections in each animal (n = 6) (Fig. 1, C–E, and Supplemental Fig. 1). Colabeled cells were all confined within the injection site except for one animal where a few PHA-L+kisspeptin cells were also identified in the contralateral ARN. This could be due to tracer uptake by dendrites that extend across into the injection site (22). As shown in the series of drawings in Fig. 1, I–N, PHA-L-labeled fibers from the rARN were identified in many limbic brain regions in good agreement with previous ARN anterograde tracing studies (28–31). Dual-labeled PHA-L+kisspeptin fibers (Fig. 1, F–H) were observed in several preoptic nuclei, including median preoptic nucleus (MEPO), AVPV, PVpo, medial preoptic nucleus (MPN), as well as the adjacent lateral preoptic area (Fig. 1, I–K). Dual-labeled fibers were also found in bed nuclei of the stria terminalis (BST), periventricular nucleus, paraventricular nucleus (PVN), lateral hypothalamic area (LHA), dorsomedial nucleus (DMN), contralateral ARN, and posterior hypothalamic area (Fig. 1, I–M). In the midbrain, PHA-L+kisspeptin fibers were found only in the periaqueductal gray (PAG) (Fig. 1N). Notably, colabeled fibers were absent in several brain regions where PHA-L-labeled fibers were found; the lateral septum (LS), anterodorsal preoptic nucleus, anterior hypothalamic area, paraventricular thalamic nucleus (PV), ventromedial nucleus (VMN), ME, and premammillary nucleus. Animals with PHA-L deposited in cARN showed fibers predominantly in the caudal

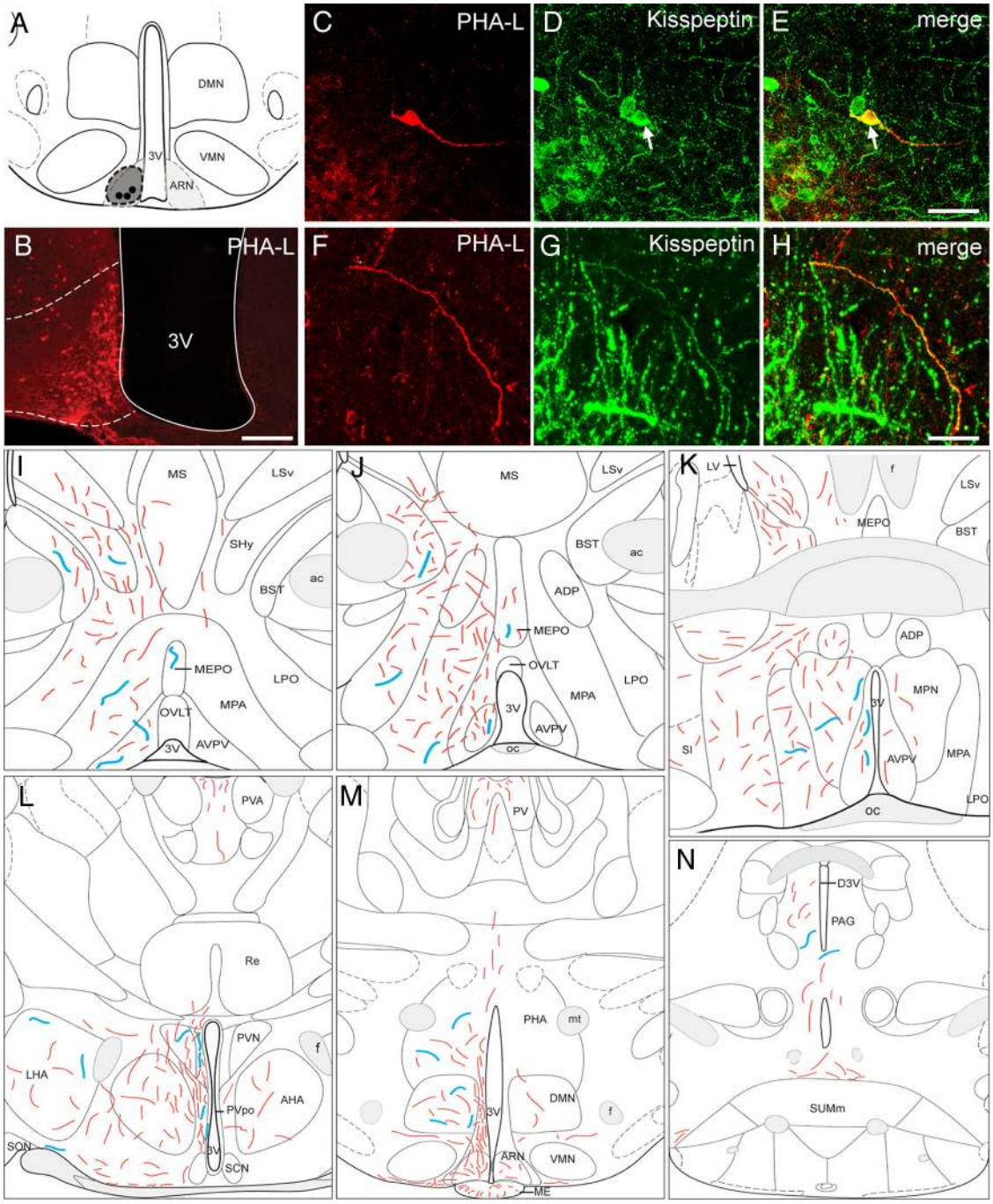

FIG. 1. Anterograde tracing of ARN kisspeptin neuronal projections. A, Schematic diagram showing the extent of PHA-L injection into the rARN nucleus (*dark gray shading*) and distribution of kisspeptin cells (*filled circles*) that have taken up PHA-L. B, Photomicrograph of another mouse showing injection of PHA-L in rARN. C–E, Dual immunofluorescence showing PHA-L (*red*) in a kisspeptin cell (*green*) in the ARN. F–H, Confocal image of anterogradely labeled PHA-L fiber (*red*) that also contains kisspeptin (*green*) in the medial preoptic area (MPA); this projection image was generated from a stack of eight 1-μm-thick confocal images. I–N, Coronal sections of mouse brain arranged from rostral to caudal illustrating the distribution of PHA-L (*red*) and dual-labeled PHA-L+kisspeptin fibers (*blue*) in one representative animal. *Scale bars*: 100 μm (B), 25 μm (C–E), and 15 μm (F–H). ADP, Anterodorsal preoptic nucleus; AHA, anterior hypothalamic area; BST, bed nucleus of stria terminalis; LPO, lateral preoptic area; LSv, LS (ventral part); MS, medial septal nucleus; OVLT, organum vasculosum of the lamina terminalis; PHA, posterior hypothalamic area; PVA, PV (anterior part); Re, reuniens thalamic nucleus; SCN, suprachiasmatic nucleus; SHy, septohypothalamic nucleus; SuM, supramammillary nucleus; 3V, third ventricle; ac, anterior commissure; f, fornix; mt; mammilothalamic tract; oc, optic chiasm.

hypothalamus, including the caudal PVpo, PVN, LHA, DMN, posterior hypothalamic area, and PAG with dual-labeled PHA-L+kisspeptin fibers identified in all these areas except the LHA.

Mice in which the PHA-L injection was not located in the ARN (n = 10), for example in the VMN or supramammillary nucleus, did not exhibit any dual-labeled PHA-L+kisspeptin cells or fibers. The omission of the

PHA-L antisera from the immunocytochemistry protocol resulted in a complete absence of staining.

RP3V

A large number of experiments in which PHA-L was injected into the AVPV and PVpo failed to result in uptake of PHA-L into kisspeptin neurons in these regions. To overcome this problem, we used BDA injection with a pressure syringe system. This resulted in a greater spread of anterograde tracer and also uptake of BDA into RP3V kisspeptin neurons. Of 20 animals receiving BDA injections, two had BDA deposition involving the AVPV (Fig. 2A and Supplemental Fig. 2A) and three the PVpo (Fig. 2B and Supplemental Fig. 2B). Examination of RP3V sections with tracer deposition (n = 5) revealed 4 ± 0.2 kisspeptin cell bodies labeled with BDA per three sections (Fig. 2, C–E, and Supplemental Fig. 2, A and B). Mice in which the BDA injection missed the RP3V, for example in the MEPO or MPN, did not exhibit any dual-labeled BDA+kisspeptin cells or fibers.

The distribution of BDA-labeled fibers after injection into the AVPV was very similar to that described previously in the rat (32) and is depicted in Fig. 2, J and K. Dual-labeled BDA+kisspeptin fibers originating from the AVPV (Fig. 2, F–K) were detected in the ventral LS, BST, MEPO, medial preoptic area, periventricular nucleus, DMN, ARN, and PV (anterior part). Dual-labeled BDA+kisspeptin fibers originating from the PVpo were found in the same regions as AVPV injected mice with the exception of the LS, BST, MEPO, and the addition of the subfornical organ and ventrolateral PAG (Fig. 2, L–O).

Experiment 2. Retrograde tracing of ARN kisspeptin neuronal projections to the rPOA

The results above indicated that ARN kisspeptin neurons projected widely in the brain and that this included the rPOA, where the GnRH neuron cell bodies are located. To verify this potentially important pathway, retrograde tracing experiments were undertaken. A total of eight OVX female mice were given FG injections into the rPOA encompassing the vertical limb of the diagonal band of Broca, caudal medial septum, and MEPO (Fig. 3A). Six of the FG depositions were midline within the rPOA (Fig. 3A and Supplemental Fig. 3A), with two being lateral (Fig. 3B). Immunostaining of every third rPOA section for FG and GnRH demonstrated that FG had been deposited within the vicinity of GnRH neuron perikarya in all eight mice (Fig. 3, A and C).

To enable dual-labeling with the polyclonal rabbit anti-FG antiserum, we used a new polyclonal sheep antiserum directed against kisspeptin-10. The distribution of immunoreactivity with the AC024 antibody (Fig. 3, D and J) was identical to that found with the AC566 antisera. Control experiments, including the preadsorption of AC024 with kisspeptin-10 peptide and staining of hypothalamic brain sections from adult female *Kiss1* knockout mice, resulted in a complete absence of kisspeptin staining.

FG immunoreactivity was evident as an intracytoplasmic punctate label (Fig. 3, E and K). After FG injections into the rPOA, retrogradely labeled neurons were detected throughout the hypothalamus, including the BST, AVPV, PVpo, MPN, DMN, VMN, LHA, and ARN, as noted previously in the rat (33, 34). Dual-labeled FG+kisspeptin neurons were found bilaterally in the ARN of midline FG-injected mice and predominantly unilaterally in lateral FG-injected mice. Kisspeptin neurons were observed throughout the ARN in the same pattern as described previously (3) with mean numbers of 10.4 ± 1.3 cells/section in the rARN and 10.3 ± 1.9 cells/section in the cARN. The great majority (89%) of dual-labeled ARN+FG kisspeptin neurons were located in the rARN (3.2 ± 0.5 dual-labeled cells/section) with only small numbers detected in the cARN (0.4 ± 0.2). Although mice receiving midline FG injections had dual-labeled FG+kisspeptin cells bilaterally in the ARN, the two mice with lateral FG injection exhibited only ipsilateral dual-labeled cells. In total, 21 ± 2% of ARN kisspeptin neurons were found to contain FG, indicating that they projected to the rPOA (Fig. 4B).

Experiment 3. Retrograde tracing of RP3V kisspeptin projections to the ARN

Alongside previous correlative evidence (6, 35), the anterograde tracing studies highlighted the possibility that RP3V kisspeptin neurons send a major projection to the ARN. To verify this projection, mice were given FG injections into the ARN. Six such mice were found to have discrete unilateral FG injections within the rARN (n = 2) (Fig. 3, G and I) or cARN (n = 4) (Fig. 3H and Supplemental Fig. 3B).

FG-labeled cell bodies were identified throughout the forebrain in a regionally discrete manner with the great majority located ipsilateral to the injection site. Numerous FG-labeled cell bodies were detected in the LS, BNST, MEPO, AVPV (Fig. 3K), PVpo, MPN, PVN, anterior hypothalamic area, medial amygdala, and ARN, whereas lower numbers were detected in the PV (anterior part), LHA, and DMN (data not shown). This distribution of retrogradely labeled cells is in good agreement with previous observations (30) and was similar in all six animals.

Analysis of dual-labeled sections revealed numerous kisspeptin neurons with FG (Fig. 3, J–L) in the AVPV (Fig. 4C) and PVpo (Fig. 4E). Most of the FG+kisspeptin neurons were found ipsilateral to the injection site, representing 44 ± 6% of AVPV (Fig. 4, C and D) and 43 ± 6% of

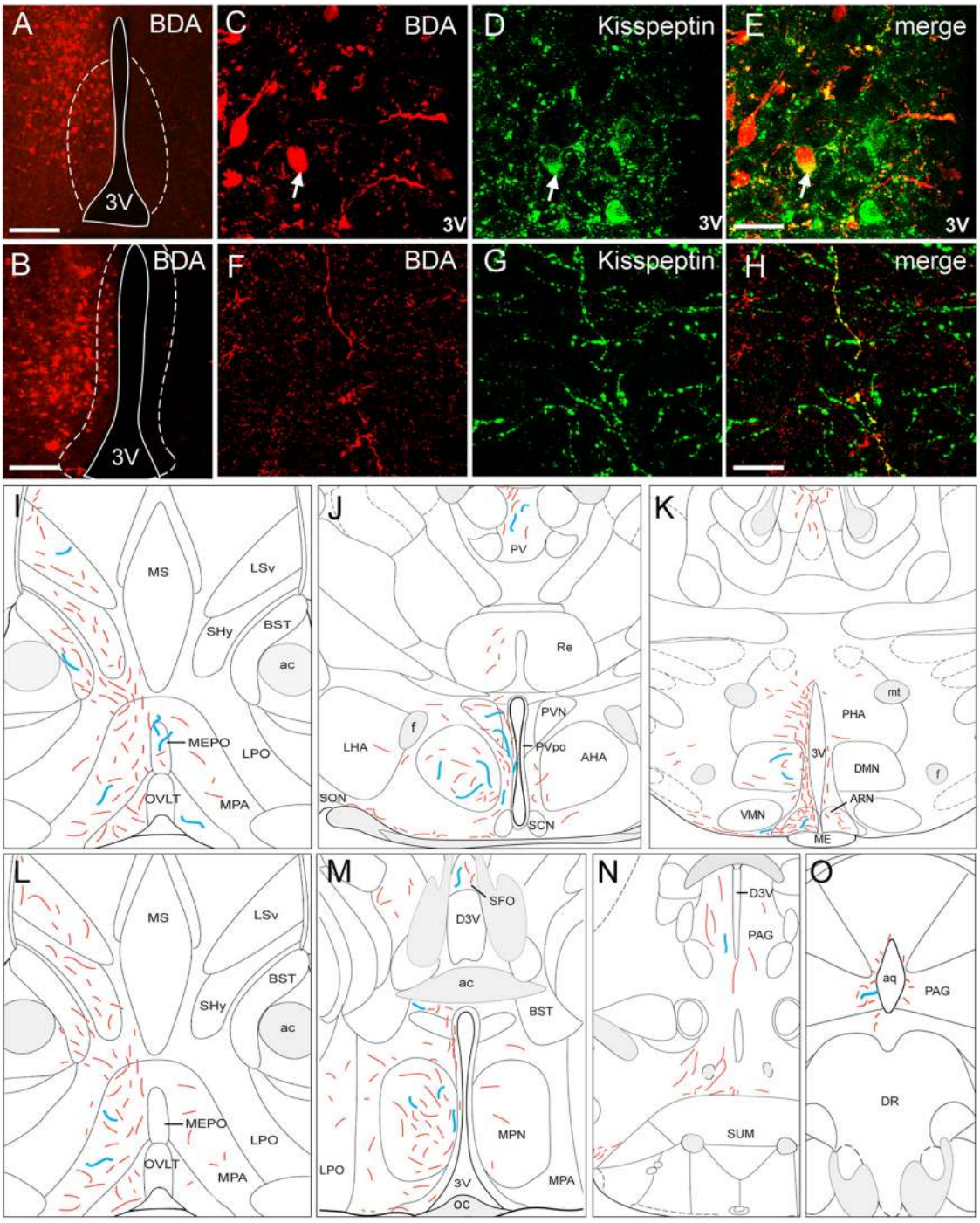

FIG. 2. Anterograde tracing of RP3V kisspeptin neuronal projections. A and B, Low-power photos showing BDA deposition into the AVPV (A) and PVpo (B) and adjacent areas of two different mice. C–E, High-power view of a PVpo neurons containing BDA (*arrow*, *red*) as well as kisspeptin (*arrow*, *green*). F–H, Confocal image of anterogradely labeled BDA fiber (*red*) that also contains kisspeptin (*green*) in the ARN nucleus. I–K, Schematic diagrams showing the distribution of single-labeled BDA (*red*) and dual-labeled BDA+kisspeptin fibers (*blue*) in the mouse with AVPV BDA deposition shown in A. L–O, Schematic diagrams showing the distribution of single-labeled BDA (*red*) and dual-labeled BDA+kisspeptin fibers (*blue*) in the mouse with BDA deposition in the PVpo (B). *Scale bars*: 200 μm (A and B), 20 μm (C–E), and 15 μm (G–I). AHA, Anterior hypothalamic area; ARN, arcuate nucleus; BST, bed nucleus of stria terminalis; DMN, dorsomedial nucleus; DR, dorsal raphe nucleus; D3V, dorsal third ventricle; LHA, lateral hypothalamic area; LPO, lateral preoptic area; LSv, lateral septal nucleus (ventral part); ME, median eminence; MEPO, median preoptic area; MS, medial septal nucleus; MPA, medial preoptic area; MPN, medial preoptic nucleus; OVLT, organum vasculosum of lamina terminalis; PAG, periaqueductal gray; PHA, posterior hypothalamic area; PV, paraventricular nucleus thalamus; PVN, paraventricular nucleus hypothalamus; PVpo, periventricular preoptic nucleus; Re, reuniens thalamic nucleus; SCN, suprachiasmatic nucleus; SFO, subfornical organ; SHy, septohypothalamic nucleus; SON, supraoptic nucleus; SuM, supramammillary nucleus; VMN, ventromedial nucleus; 3V, third ventricle; ac, anterior commissure; aq, cerebral aqueduct; f, fornix; mt; mammilothalamic tract; oc, optic chiasm.

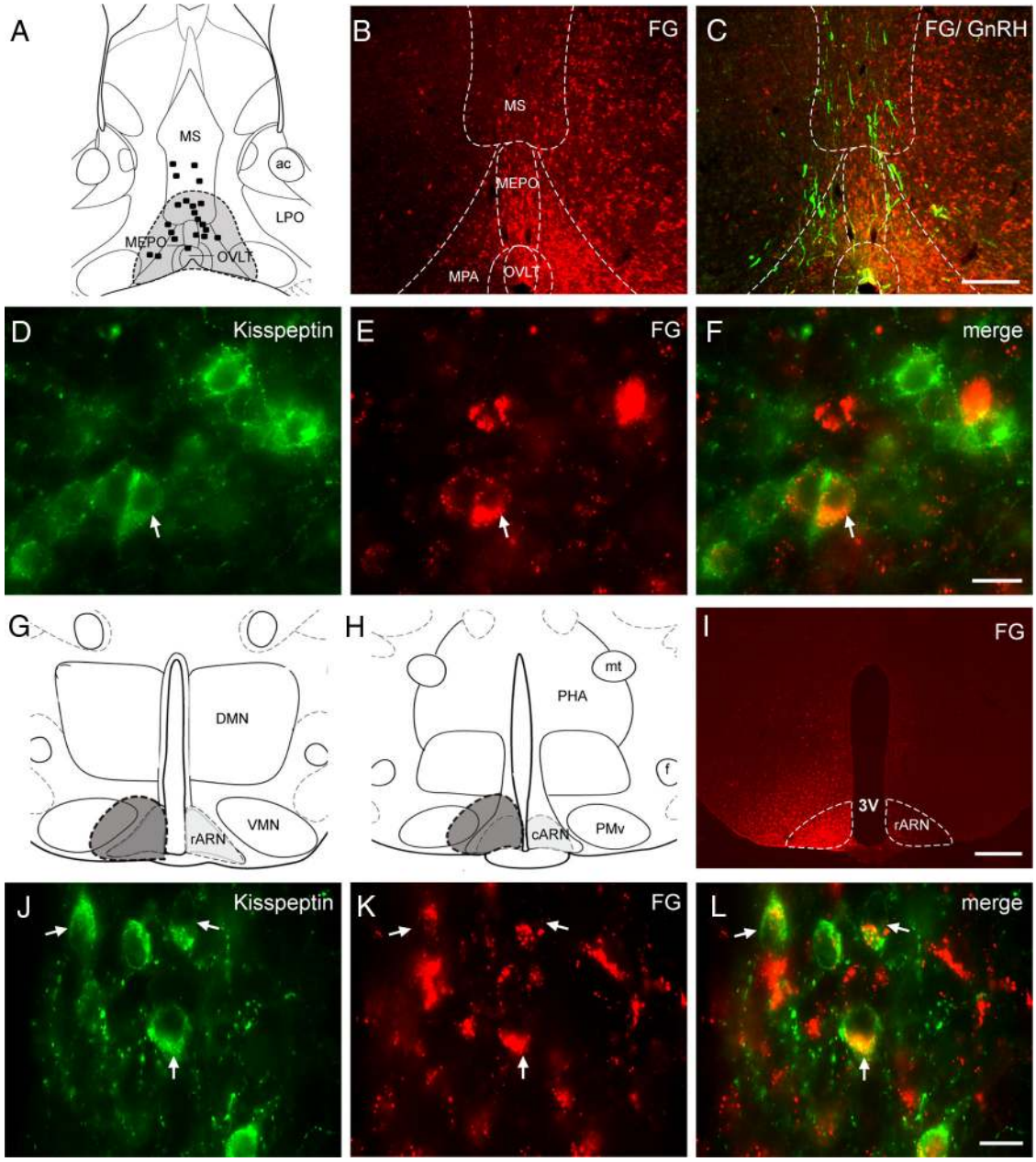

FIG. 3. Retrograde tracing of kisspeptin neurons from the rPOA and ARN. A, Schematic showing extent of a midline FG deposition into the rPOA (*gray-shaded area*), *filled squares* represent the distribution of GnRH neurons. B and C, Immunolabeling indicating lateral FG injection site (*red*) (B) and its relationship to rPOA GnRH neurons (*green*) (C). D–F, Photomicrograph of an ARN kisspeptin neuron (*green*, indicated by *arrow*) with FG uptake (*red and yellow*, *arrow*). G and H, Diagrams showing FG injection sites (*gray*) in the rostral (G) and caudal ARN (H). I, Low-magnification photomicrograph indicating FG injection site in rARN. J–L, Kisspeptin cells in the PVpo (*green*, *arrows*) colabeled with FG (*red and yellow*, *arrows*). *Scale bars*: 50 μm (B–C), 20 μm (D–F and J–L), and 500 μm (I). PMv, Premammillary nucleus (ventral part); 3V, third ventricle; ac, anterior commissure; mt; mammilothalamic tract; LPO, lateral preoptic area; MPA, medial preoptic area.

PVpo kisspeptin neurons (Fig. 4, E and F) compared with 25 ± 8 and 29 ± 10% of kisspeptin neurons in the contralateral AVPV and PVpo, respectively (Fig. 4, C–F). No differences were detected between mice given rARN or cARN FG injections.

One concern with near-midline FG injections used here is the possibility of FG diffusion into the cerebrospinal fluid that might then be randomly taken up by periventricular kisspeptin neurons. To examine this, FG was injected directly into the third ventricle of two mice. In none of these mice was FG found in kisspeptin neurons.

Experiment 4. Retrograde tracing of kisspeptin neuronal projections into regions outside the BBB

Our present and previous (4, 35) observations suggest that kisspeptin neurons in the mouse do not project to the external zone of the ME in the mouse. However, it is possible that kisspeptin neurons do innervate the ME, but that

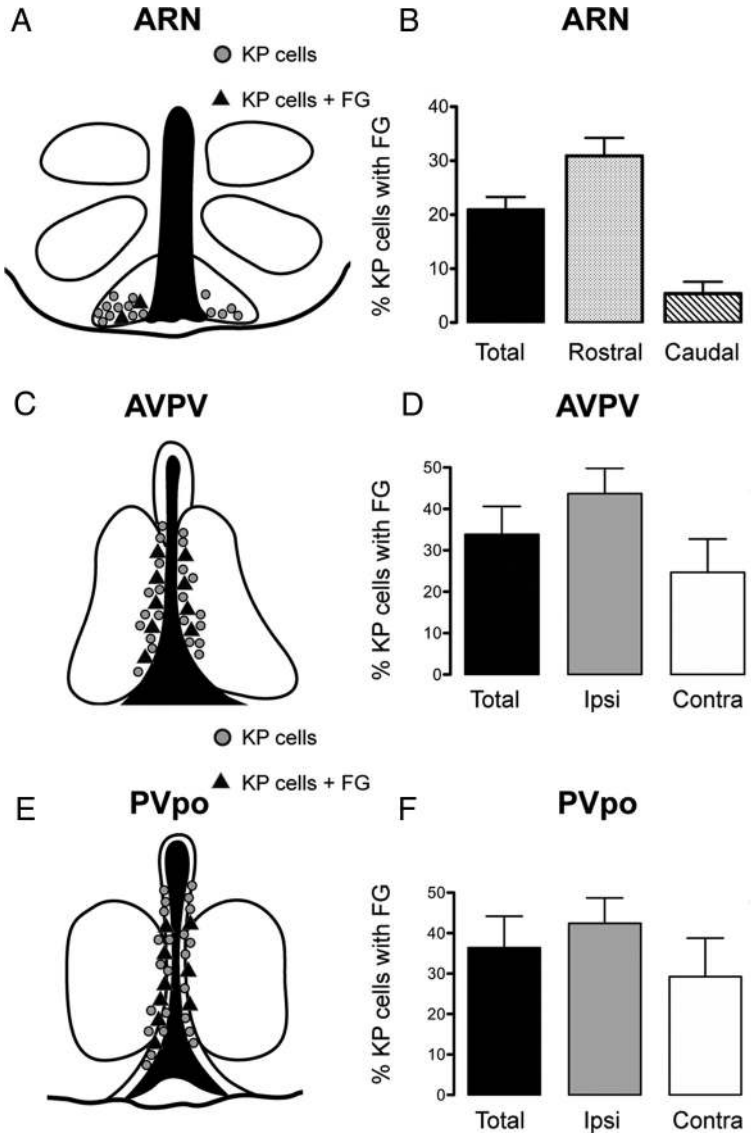

FIG. 4. Quantification of kisspeptin neurons retrogradely labeled from rPOA and ARN. A, Distribution of ARN kisspeptin neurons retrogradely labeled from FG injections into the rPOA. B, Histogram showing mean (±SEM) percentage of kisspeptin neurons with FG in the total ARN, rARN, and cARN. C–E, Distribution of kisspeptin neurons in AVPV (C) and PVpo (E) after unilateral FG injections into the ARN. D and F, Histograms showing the percentage of total, ipsilateral (Ipsi), and contralateral (Contra) kisspeptin neurons colabeled with FG in AVPV (D) and PVpo (F).

the fibers in the ME have a kisspeptin peptide content too low for detection by immunocytochemistry. To address this issue, we used the ability of ip FG to be taken up by nerve terminals lying outside the BBB to reevaluate whether kisspeptin neurons may project to the external zone of the ME. Both intact and OVX mice were examined to ensure that maximal numbers of kisspeptin neurons were observed in the RP3V and ARN, respectively, and to ensure that there was no major effect of gonadal steroids on FG uptake at the ME (36).

Cell bodies containing FG were detected in all regions known to have neurons that project outside the BBB, including the PVpo, supraoptic nucleus, PVN, and ARN. Kisspeptin neurons were identified in the RP3V and ARN in the normal distribution. Compared with intact mice, OVX animals exhibited 3-fold increase in kisspeptin cell number in the ARN ($P < 0.01$) (Table 1), a 40% decrease in kisspeptin cell number in the PVpo, and no difference in the AVPV. Dual-labeling revealed that no kisspeptin cells located anywhere in the hypothalamus contained FG in any of the five OVX or five intact animals examined (Fig. 5, A–F). In contrast, the majority of GnRH neurons were found to have FG in both experimental groups, 62 ± 8% of rPOA GnRH neurons in OVX animals compared with 74 ± 5% in sham-operated animals (Fig. 5, G–I).

Discussion

Using tract tracing, we demonstrate here the projection patterns of the ARN and RP3V kisspeptin cell populations in the female mouse brain (summarized in Fig. 6). In general, the kisspeptin neurons of the ARN are found to proj-

TABLE 1. Mean number (±SEM) of single- and dual-labeled kisspeptin neurons in the AVPV, PVpo and ARN, and GnRH neurons in the rPOA of sham *vs.* OVX animals after ip FG injection

Animal	No. kisspeptin neurons/section			KP + FG	No. GnRH neurons/ section rPOA	No. GnRH + FG rPOA	% GnRH + FG rPOA
	AVPV	PVpo	ARN				
Sham ($n = 5$)	44.3 ± 5.8	68.6 ± 6.8[a]	6.9 ± 1.7[b]	0	34.6 ± 1.3[a]	25.5 ± 1.7	73.7 ± 4.4
OVX ($n = 5$)	50.2 ± 4.3	43.6 ± 4.3	20.1 ± 3.1	0	20.9 ± 1.6	12.8 ± 1.5	62.0 ± 7.4

[a] $P < 0.05$ compared with OVX.
[b] $P < 0.01$ compared with OVX.

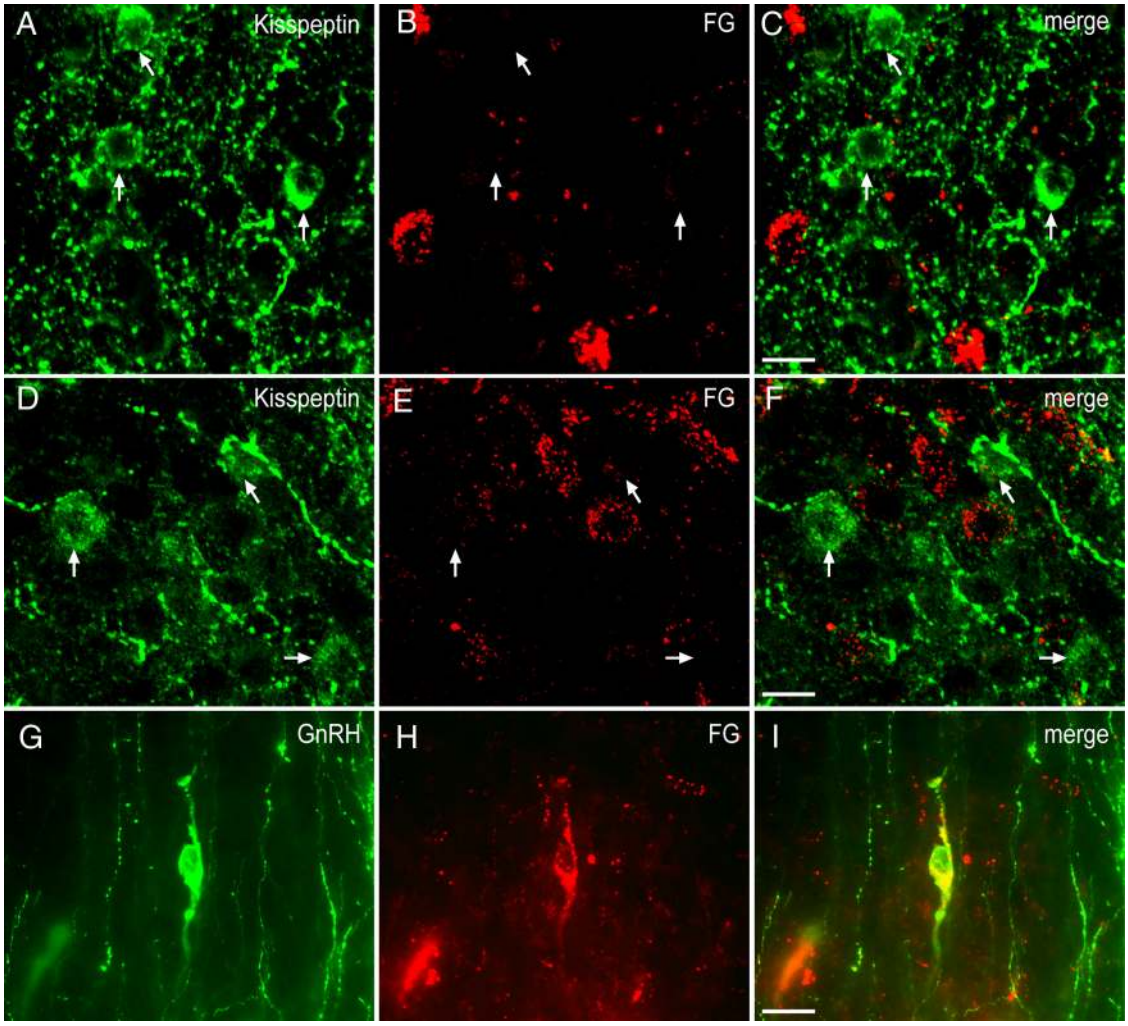

FIG. 5. Kisspeptin neurons do not project to regions outside the BBB. A–F, Confocal images showing kisspeptin neurons in the AVPV (A) and ARN (D) that do not contain FG (B and E); overlapping images in C and F. These images were generated from a stack of five 1.14-μm-thick focal planes. G–I, Representative photomicrograph of a dual-labeled GnRH neuron (*green*) with FG (*red*). *Scale bars*: 15 μm (A–C), 10 μm (D–F), and 20 μm (G–I).

ect widely within both medial and lateral aspects of the hypothalamus and associated limbic structures, whereas RP3V kisspeptin neurons have a more medial projection pattern. The diversity of brain regions targeted by kisspeptin neurons suggests their involvement in regulating multiple circuits outside that of the GnRH neuronal network. Of particular interest, we note 1) a substantial kisspeptin projection pathway from RP3V kisspeptin neurons to the ARN, 2) innervation of the rPOA by only rARN kisspeptin neurons, and 3) no evidence for any RP3V or ARN kisspeptin neurons targeting the external zone of the ME.

In undertaking these studies, we sought to use the gold-standard anterograde tracer PHA-L. Although troublesome in its variable uptake and limited spread, this tracer provides definitive evidence of neuronal projections (37, 38). We were able to use this tracer effectively in our ARN anterograde tracing studies but failed to achieve any substantial uptake into kisspeptin neurons when injected into the RP3V. The reasons for this remain unknown. To achieve our goal of tracing the RP3V kisspeptin neurons, we turned to using BDA as an anterograde tracer and, possibly because of the wider diffusion after pressure injection, found this to be effective. The ARN anterograde labeling study had to be performed in OVX mice, to enable us to see ARN kisspeptin cell bodies, and it is possible that the innervation pattern of these neurons may be altered by OVX. In all cases, it should be noted that the accurate placement of tracer is challenging in the mouse brain with, typically, less than 30% of injections being correctly located within the boundaries of the target in our hands. Even with correct injections sites, we acknowledge that relatively few kisspeptin neurons were labeled in each mouse. Our analysis indicates that, on average, only three or four kisspeptin cells were labeled in each brain section examined. Taking account of the injection volume, we

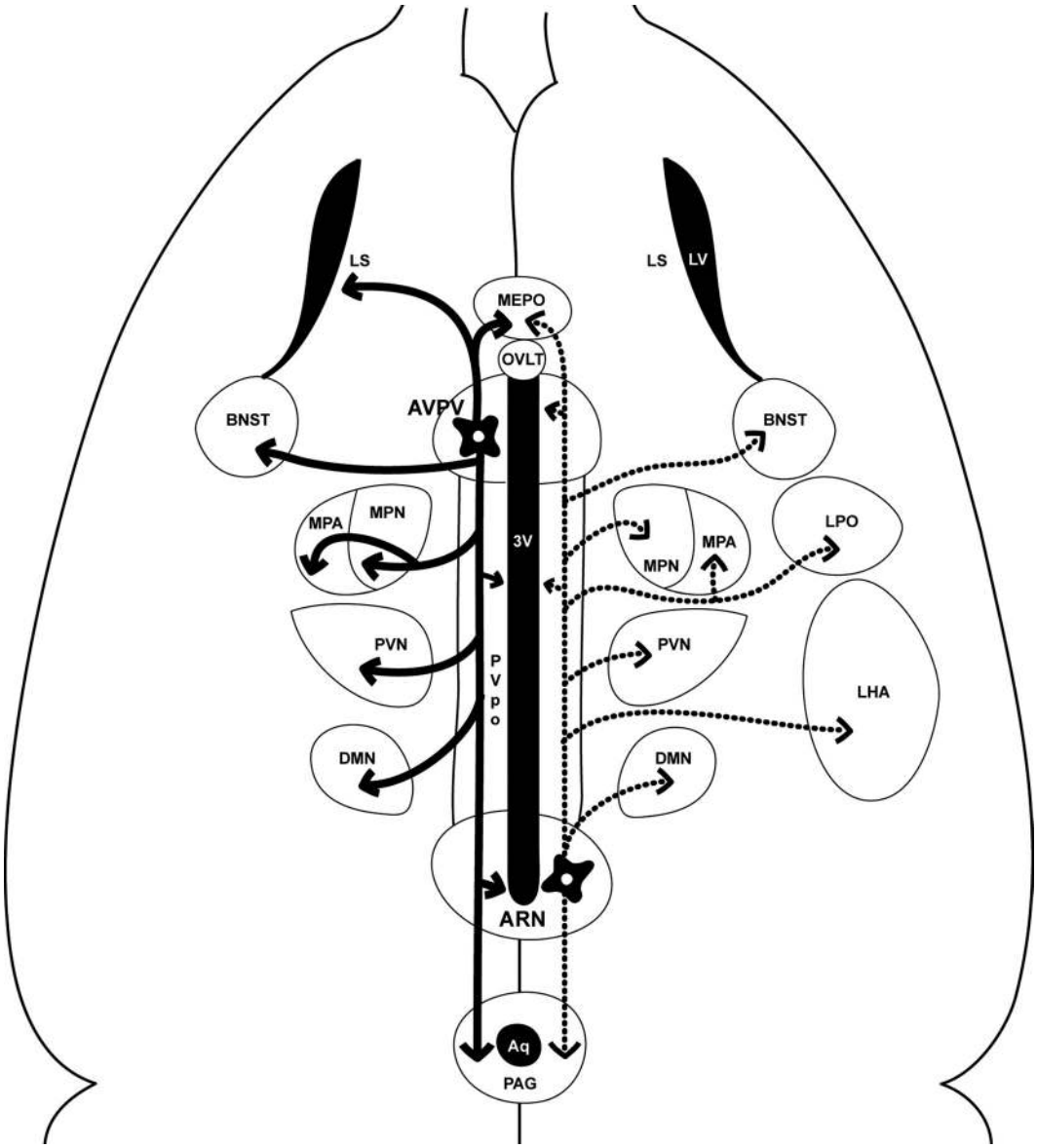

FIG. 6. Schematic, flat-map horizontal view of the female mouse brain showing the organization of kisspeptin neuronal projections. RP3V kisspeptin neuron projections are represented by AVPV neuron on the *left*, whereas ARN kisspeptin neuron projections are indicated on the *right* (*dotted line*). MPA, Medial preoptic area; aq, cerebral aqueduct; LPO, lateral preoptic area; OVLT, vascular organ of lamina terminalis; 3V, third ventricle.

estimate that approximately 20 kisspeptin neurons were labeled in the ARN and RP3V of each mouse (Supplemental Figs. 1 and 2). Nevertheless, despite these populations being sampled at random, we found the projections from individual ARN- or RP3V-injected mice to be highly consistent. This leads us to suspect that the mapping described here is a reasonable approximation of both ARN and RP3V kisspeptin neuron projections.

The overall pattern of PHA-L fiber staining achieved after injection in the ARN is in good agreement with other rodent studies (28, 31, 39). Although dual-labeled kisspeptin+PHA-L fibers were not found in all brain regions with PHA-L fibers, they were observed in many hypothalamic nuclei as well as the BST and PAG (Fig. 6). These data suggest that ARN kisspeptin neurons are involved in modulating multiple neuroendocrine and hypothalamic neuronal networks. One curious correlate of this finding is that there is rather little correspondence between the locations of ARN kisspeptin fibers and cells transcribing *Gpr54* in the female mouse (40). For instance, *Gpr54* is highly expressed in the dentate gyrus of the hippocampus, supramammillary nucleus, and anteroventral nucleus of thalamus, but none of these brain regions were found to have kisspeptin fibers here or in previous studies (3). Conversely, many hypothalamic brain regions were found to have kisspeptin fibers but no *Gpr54* gene expression. This suggests 1) that the fibers observed are *en passage* to another brain region, 2) that the Gpr54-lacZ knockin trans-

genic mouse line used for the Gpr54 mapping studies is not sufficiently sensitive, and/or 3) that kisspeptin can use another receptor for modulating non-GnRH neuronal networks. Of relevance, a recent study has demonstrated electrophysiological effects of kisspeptin and the presence of Gpr54 mRNA in the ARN of the mouse (16), indicating that the receptor is expressed in the ARN.

Given the proposed importance of ARN kisspeptin neurons in regulating the activity of GnRH neurons (5, 7–9), projections of ARN kisspeptin neurons to the GnRH neuron cell bodies in the rPOA and terminals in the ME are of special interest. We find here that approximately 20% of ARN kisspeptin neurons send projections to the rPOA and that, intriguingly, these cells are located almost exclusively in the rARN. There is presently little information on potential differences between rARN and cARN kisspeptin neurons in rodents, although a previous study in rats has shown that estrogen receptor α-expressing neurons that project to the rPOA are also concentrated in the rARN (33). In addition, lesions of the rARN have been found to be the most effective in suppressing LH secretion (41). These results raise the possibility that rARN kisspeptin neurons may innervate GnRH neuron cell bodies. Recent studies using the coexpression of neurokinin B and kisspeptin to identify ARN kisspeptin neurons have reported only a very few dual-labeled fibers in the vicinity of the rat rPOA (31, 39). Although positive results from those studies are useful, they are reliant upon detectable amounts of both neurokinin B and kisspeptin peptides in the axons of ARN and can suffer from false negative errors.

We find here very little support for the notion that ARN kisspeptin neurons send projections to GnRH nerve terminals in the external layer of the ME. In our previous studies (35), we noted a lack of kisspeptin-immunoreactive fibers in the external zone of the ME of the mouse. This result is not dissimilar to work in the rat (39, 42) and primate (43), where the vast majority of kisspeptin fibers are found in the internal zone of the ME. It remains, however, that kisspeptin is able to modulate GnRH release from the ME *in vitro* in the mouse and sheep (14, 15). Thus, we considered the possibility that kisspeptin peptide levels are just too low in axons and terminals of the external ME to be detected by immunocytochemistry. As such, we used peripheral injection of FG to examine whether kisspeptin neurons have terminals outside the BBB in the external zone of the ME. We find no evidence for FG uptake by any kisspeptin neurons, regardless of their location in the RP3V or ARN, in either intact or OVX mice. In contrast, in the same mice, the majority of GnRH neurons contained FG. Together, these data indicate that ARN and RP3V kisspeptin neurons do not project axons to GnRH nerve terminals in the external zone of the ME. This leaves open the question of how kisspeptin might influence secretion from GnRH nerve terminals. One possibility, raised by several investigators, is that kisspeptin may be released from terminals in the internal zone of the ME and act through volume transmission to eventually reach and control GnRH release in the external zone.

The RP3V kisspeptin neurons provide inputs to a variety of midline hypothalamic nuclei in addition to the LS and BST (Fig. 6). As suggested for the ARN kisspeptin neurons, it seems likely that the RP3V kisspeptin cells are also involved in modulating the activity of multiple neuronal circuits. Previous studies had indicated a close relationship between the numbers of kisspeptin neurons in the RP3V and density of kisspeptin fibers in the ARN (11), and AVPV neurons are known to send a substantial projection to the ARN in rats (32). Anterograde tracing studies undertaken here demonstrate that RP3V kisspeptin neurons project to the ARN, and retrograde labeling identified that approximately 40% of RP3V kisspeptin neurons innervated both rostral and caudal elements of the ARN bilaterally. Hence, as suspected, these observations demonstrate a major projection of RP3V kisspeptin neurons to the ARN.

Evidence to date indicates that RP3V kisspeptin neurons innervate GnRH neuron cell bodies in a direct manner; the number of kisspeptin appositions on GnRH neurons are directly proportional to RP3V kisspeptin cell number (35), kisspeptin/tyrosine hydroxylase colabeled fibers, that can only come from the RP3V, are apposed to GnRH neurons (44), and electrical activation of the RP3V evokes kisspeptin-mediated excitation of GnRH neurons (45). Although it remains unclear whether RP3V kisspeptin neurons projecting to GnRH neurons have collaterals innervating other brain regions, the present data suggest that the RP3V kisspeptin neuron population as a whole is well positioned to coordinate the activity of multiple neuroendocrine axes. The great majority of kisspeptin neurons in the RP3V are estrogen receptive (27) and have their Kiss1 mRNA expression elevated at proestrus (27, 46). Equally, the firing rates of RP3V neurons are increased at proestrus (47). Hence, it is possible that RP3V kisspeptin neurons coordinate the steroid-dependent plasticity of GnRH and other neuronal cell types in the hypothalamus. Of particular relevance, both dopamine (17) and proopiomelanocortin (16) neuronal populations in the ARN are suspected to be regulated directly by kisspeptin.

The present study describes the projection patterns of ARN and RP3V kisspeptin neurons in mice and highlights the wide number of neuronal circuits likely to be regulated by kisspeptin. In terms of the regulation of GnRH neurons, the experiments provide support for the possible innervation of GnRH neuron cell bodies/dendrites by rARN kiss-

peptin neurons. In contrast, we find no evidence that any kisspeptin neuron population projects to the GnRH nerve terminals in the external zone of the ME. The findings also emphasize the complexity of the kisspeptin neuronal system and suggest that it is too simplistic to consider whole populations of kisspeptin neurons only in terms of the GnRH neuronal network. For example, the kisspeptin neurons located in the caudal ARN project widely within the limbic brain but appear to have no direct inputs to GnRH neurons. Finally, the diverse and partially overlapping projections of both kisspeptin populations within the hypothalamus indicate the possibility of differential roles in the central coordination of steroid-dependent neuroendocrine circuits.

Acknowledgments

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