Gonadotropin-Releasing Hormone Neurons Extend Complex Highly Branched Dendritic Trees Outside the Blood-Brain Barrier

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GnRH neurons project axons to the median eminence to control pituitary release of gonadotropins and, as such, represent the principal output neurons of the neuronal network controlling fertility. It is well established that the GnRH neurons exhibit a simple bipolar morphology with one or two long dendrites. Using adult GnRH-green fluorescent protein transgenic mice and juxtacellular cell filling, we report here that a subpopulation of GnRH neurons located in the rostral preoptic area exhibit extremely complex branching dendritic trees that fill the organum vasculosum of the lamina terminalis (OVLT). The dendritic nature of these processes was demonstrated at both light and electron microscopic levels by the presence of spines, dendritic ultrastructure, and synapses. Further, electrophysiological recordings showed that GnRH neurons were excited by glutamate as well as kisspeptin puffed onto their dendrites located within the OVLT. Using iv injection of horseradish peroxidase, a molecule unable to penetrate the blood-brain barrier (BBB), we show that GnRH neuron cell bodies and dendrites within 100 μm of the OVLT reside outside the BBB. Approximately 85% of GnRH neurons in this area express c-Fos at the time of the GnRH surge. These observations demonstrate that GnRH neurons extend complex, highly branched dendritic trees beyond the BBB into the OVLT, where they will be able to sense directly molecules circulating in the bloodstream. This indicates a new mechanism for the modulation of GnRH neurons that extends considerably the range of factors that are integrated by these neurons for the control of fertility. **(*Endocrinology* 152: 3832–3841, 2011)**

The brain is protected from the constituents of the bloodstream by tight endothelial junctions forming the blood-brain barrier (BBB). The only exceptions to this arrangement are found in the circumventricular organs, including the subfornical organ, area postrema, median eminence, and organum vasculosum of the lamina terminalis (OVLT); specialized sites at which direct communication occurs between the blood stream and neuronal and glial elements. A substantial amount of work has shown that cells in circumventricular organs, rich in a variety of neuropeptide and cytokine receptors, have key roles in sensing systemic toxins, including pyrogens, and molecules related to body fluid homeostasis (1–4). However, relatively little is known about roles of circumventricular organs outside these functions (3, 5).

GnRH neurons are the final output neurons of the hypothalamus that control fertility in all mammals. The function of GnRH neurons is reflected in part by their anatomy, because despite having their cell bodies scattered throughout the basal forebrain, most GnRH neurons project axons to the external zone of the median eminence from where they secrete GnRH into the portal vasculature to control pituitary gonadotropin release (6–8). One curious and unexplained feature of the GnRH neuronal system, however, has been that of the role of the very high density of GnRH fibers found within the

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For editorial see page 3589

Abbreviations: ACSF, Artificial cerebrospinal fluid; BBB, blood-brain barrier; b.w., body weight; GFP, green fluorescent protein; HRP, horseradish peroxidase; OVLT, organum vasculosum of the lamina terminalis; PSI, pounds per square inch; rPOA, rostral preoptic area; TBS, Tris-buffered saline.

OVLT (6). Although recognized for over 35 yr (9), the function of these GnRH neuron projections has remained unknown (8).

Morphological studies have shown that GnRH neurons have a very simple uni- or bipolar morphology with very long, typically unbranched dendrites (>1000 μm) that are covered in spines (10, 11). These dendrites frequently bundle with dendrites of other GnRH neurons and make close appositions that enable them to share individual synaptic input (12). Further, GnRH neuron dendrites possess active conductances and can even be the site of action potential initiation (13). These findings have led to an emerging view that GnRH neuron dendrites are the key neuronal compartment for synaptic integration and spike initiation in these cells (14). In view of the emerging importance of the GnRH neuron dendrite, it is critical to obtain a full understanding of their structure and function.

We report here the surprising observation that a subpopulation of GnRH neurons located in the rostral preoptic area (rPOA) extend extraordinarily complex processes into the OVLT. These processes exhibit ultrastructural and electrophysiological characteristics of dendrites and are shown to lie outside the BBB.

Materials and Methods

Experimental animals

GnRH-green fluorescent protein (GFP) mice (15) were housed with 12 h of light and *ad libitum* access to food and water. All experiments were approved by the University of Otago Animal Welfare and Ethics Committee.

Juxtacellular filling of GnRH neurons

GnRH-GFP mice at the age of 40–90 d were killed, and 200-μm-thick coronal brain slices containing the POA and OVLT were prepared as described previously (10). GnRH neurons were briefly identified under fluorescence using ×10 and ×40 water immersion objectives and a loose patch configuration (15–60 MΩ) was obtained under differential interference contrast optics. Patch pipettes (6–9 MΩ) were pulled on a horizontal puller (P-97; Sutter Instrument Co., Novato, CA) using borosilicate capillaries (G150TF-3; Warner Instruments, Hamden, CT). The pipette solution (pH 7.3) contained (in mM) 135 K-gluconate, 5 NaCl, 10 HEPES, 10 EGTA, 1 $MgCl_2$, 1 $CaCl_2$, 5 MgATP, and 0.1 Na_2GTP and Neurobiotin (SP-1120; Vector Laboratories, Burlingame, CA) was added to a final concentration of 0.2%. Juxtacellular filling was achieved using voltage steps of 200 mV and 60-msec duration delivered at 10 Hz through an AxoClamp 2B amplifier (Axon Instruments, Sunnyvale, CA) for 20–45 min. After filling, slices were fixed in 4% paraformaldehyde (pH 7.6) at 4 C for 12 h. After washing in Tris-buffered saline (TBS) (pH7.6), slices were incubated with streptavidin-Alexa Fluor 568 (Molecular Probes, Eugene, OR) in TBS containing 0.3% Triton X-100 and 0.25% BSA at room temperature for 4 h and subsequently rinsed and mounted.

Confocal analysis

Filled GnRH neurons were imaged using a Zeiss LSM 510 confocal microscope with LSM 510 software (version 3.2; Zeiss, Oberkochen, Germany). Image stacks were acquired at 0.9-μm intervals with a 40x/1.3 Plan Neofluar objective using ×3 zoom. Images are shown as maximum intensity projections or single plane images. Images were subsequently montaged together and adjusted for contrast and brightness in Photoshop CS4; reconstructions were manually drawn using Illustrator CS4 (both Adobe Systems, San Jose, CA).

Antibodies

β-3-Tubulin was used as a general neuronal marker (1:1000, MAB1195, lot HGQ01; R&D Systems, Minneapolis, MN). Dendritic markers were microtubule-associated protein 2 (1:1000, M4403, lot 111K4806; Sigma-Aldrich, St. Louis, MO), dephosphorylated heavy neurofilament (1:500, SMI32; Sternberger Monoclonals, Princeton, NJ), and kinesin-like protein 17 (1:100, ab11261, lot 721005; Abcam, Cambridge, UK). Axonal markers used were Tau1 (1:500, MAB3420, lot LV1563313; Millipore, Bedford, MA) and phosphorylated heavy neurofilament (1:500, SMI31; Sternberger Monoclonals). GFP signal was amplified by using a GFP antibody (1:5000, A6455, lot 71B1; Molecular Probes). Secondary antibodies used were goat antirabbit and goat antimouse conjugated with Alexa Fluor 488 and Alexa Fluor 568 (1:200 to 1:1000; Molecular Probes).

Immunohistochemistry

Adult GnRH-GFP mice were anesthetized with pentobarbital and transcardially perfused with 4% paraformaldehyde in phosphate buffer (pH 7.6). Sections of 30-μm thickness were cut on a freezing stage microtome and washed in TBS. Primary antibodies were incubated for 48 h at 4 C in TBS containing 0.3% Triton X-100, 0.25% BSA, and 2% normal goat serum. Secondary antibodies were incubated in the same solution for 90 min at room temperature followed by washing steps and mounting.

Transmission electron microscopy

Experiments were conducted as reported previously (12). Briefly, two diestrous female GnRH-GFP mice were anesthetized and transcardially perfused with 4% paraformaldehyde and 0.5% glutaraldehyde. Coronal sections (50 μm) were cut on a vibratome and incubated in a mixture of primary antibodies (LR5 rabbit anti-GnRH, 1:10,000, gift of R. Benoit; rabbit anti-GFP 1:2000; Molecular Probes). Secondary antibody [ultrasmall gold conjugate of f(ab')/f(ab)2 goat antirabbit, 100.366; Aurion, Wageningen, The Netherlands] incubation, and silver enhancement was performed using Aurion reagents. Tissue was osmicated with 0.5% osmium tetroxide, resin embedded, and semithin sections (4 μm) and ultrathin serial sections (60–90 nm) were cut on an ultramicrotome. After staining with 1% uranyl acetate, ultrathin sections were examined using a Philips CM100 transmission electron microscope (Philips/FEI Corporation, Eindhoven, Holland).

Electrophysiology

Slice preparation and seal acquisition was performed as described above. Spontaneous electrical activity of GnRH neurons was recorded in voltage clamp mode, and 1 mM glutamate dis-

solved in artificial cerebrospinal fluid (ACSF) was puffed onto the slice for 20 msec at a pressure of 10 pounds per square inch (PSI) using a patch pipette (6–9 MΩ) and a PicoPump (PV820; World Precision Instruments, Sarasota, FL). Likewise, 100 nM kisspeptin-10 (Metastin-10, no. 445888; Calbiochem, San Diego, CA) dissolved in ACSF was puffed onto the slice for 5 sec at a pressure of 6 PSI. Within the recording chamber, the slice was always positioned in an orientation, where the flow of ACSF (~3.1 ml/min) was away from the soma of the recorded cell, thus preventing "spill over" to nondendritic sites.

Horseradish peroxidase (HRP) injections

Six GnRH-GFP mice were deeply anesthetized with ketamine/Domitor and HRP (type IV, P8375; Sigma-Aldrich) dissolved in sterile saline (30 mg/ml) was injected iv at a dose of 15 mg/100 g body weight (b.w.). After 5 min (n = 2) or 25 min (n = 4), the animals were transcardially perfused with 4% paraformaldehyde. Brains were sectioned (30 μm), and the peroxidase was visualized using the NiDAB reaction, pseudocolored red for photographic representation. GFP signal was enhanced using a GFP antibody as described above.

c-Fos expression in GnRH neurons

LH surges were induced in five ovariectomized adult female mice as described previously (16, 17). Briefly, ovariectomized animals with sc estradiol implants were injected with estradiol benzoate (1 μg/20 g b.w.) in the morning (1100 h) of the day before the surge. In the morning (1100 h) of the day of the surge, animals received an injection of progesterone (500 mg/20 g b.w.), which yields a reliable LH surge at lights out (1800 h). At the time of the surge, blood samples were taken and animals were transcardially perfused (1730–1800 h) with 4% paraformaldehyde as described above. Immunohistochemistry for GnRH and c-Fos was performed as described (17) using a c-Fos antibody (1:10,000, sc-52; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and a GnRH antibody (1:40,000, LR1; gift of R. Benoit, Montreal, Canada). Those GnRH neuron perikarya with or without nuclear c-Fos labeling were then counted within a 100-μm radius of the OVLT and within the remaining rPOA (three sections per mouse). A RIA for LH in the blood was performed as described previously (17).

Data presentation

Values are presented as mean ± SEM unless otherwise indicated.

Results

A subpopulation of rPOA GnRH neurons exhibit highly branched dendritic trees that extend into the OVLT

The GnRH-GFP transgenic mouse line exhibits a near perfect correlation between GFP expression and GnRH immunoreactivity (15). Using an acute brain slice preparation, individual GnRH neurons of adult male GnRH-GFP mice (n = 41; 40–90 d old) were patched and juxtacellularly filled through the patch pipette with Neurobiotin. Subsequent reconstruction of confocal images from filled cells revealed the classic simple uni- or bipolar morphology of GnRH neurons located within the rPOA and medial septum as reported previously (Fig. 1, A and B) (10). Surprisingly, however, when GnRH neurons with cell bodies located adjacent to the OVLT were investigated, they were found to extend highly branched dendritic trees into the OVLT (Figs. 1 and 2).

We analyzed the trajectories of multiple GnRH neurons within single rPOA brain slices to establish the relationship of their cell bodies and dendrites to the OVLT. This showed that if a GnRH neuron cell body was located within 100 μm of the OVLT, it would in most cases (13 of 19 filled cells, 68%) extend a highly branched dendritic tree into the OVLT (Fig. 2A). We noticed, however, that simple unbranched dendrites originating from GnRH neuron cell bodies distant to the OVLT could also become highly branched if they came near the OVLT. By filling GnRH neurons throughout the rPOA, we found that 73% of dendrites located within the OVLT were branched (n = 33 dendrites) compared with 61% within a 100-μm radius of the OVLT (n = 44), 22% in the 100- to 200-μm zone surrounding the OVLT (n = 36), and less than 8% at distances beyond 200 μm (n = 25) (Fig. 2A). This means that a GnRH neuron with its cell body located within 100 μm of the OVLT would very often extend a complex dendritic tree into the OVLT. However, it was also possible for a GnRH neuron with a more distant cell body to show the same branching phenomenon if its dendrite projected in the direction of the OVLT (see red/orange neurons in Figs. 1D and 2B). Because it is not only those neurons with cell bodies adjacent to the OVLT that contribute to its dendritic innervation and limitations of the slice procedure, it is difficult to assess the total number of rPOA GnRH neurons that send dendrites to the OVLT. However, counts in adult female and male mice, respectively, showed that 27 ± 7 and 21 ± 3% of rPOA GnRH neurons have their cell bodies residing within 100 μm of the OVLT. Thus, we suspect that more than 20% of GnRH neurons in the rPOA extend complex dendritic trees into the OVLT.

Dendritic identity of GnRH neuron neurites in the OVLT

To examine the nature of GnRH neuron neurites in the OVLT, we undertook a series of dual-immunofluorescence studies using a range of established markers in adult GnRH-GFP transgenic mice (n = 12, five male and seven female). We tested the general neuronal marker β-3-tubulin (18), the dendritic markers microtubule-associated protein 2 (19), dephosphorylated heavy neurofilament (20) and kinesin-like protein 17 (21), and the axon markers axonal phosphorylated heavy neurofilament (20) and Tau1 (22). However, despite staining appropriate compartments of other neuro-

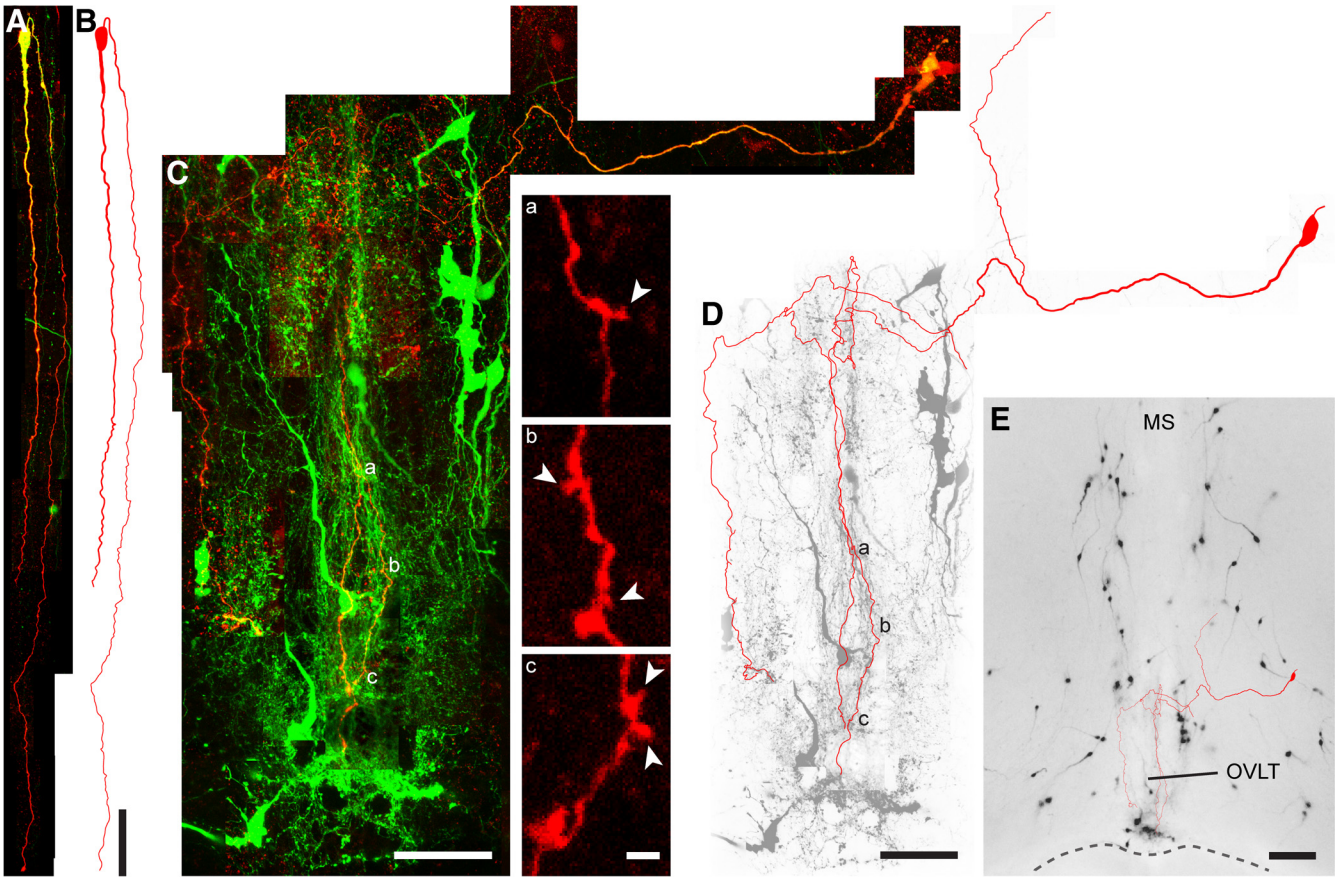

FIG. 1. Examples of Neurobiotin-filled GnRH neurons in the adult mouse brain. A, Montage of confocal stack projections of a simple bipolar GnRH neuron (*red/yellow*) in the POA. B, Camera-lucida-like schematic traced from images in A. C, Montage of confocal stack projections of a rPOA GnRH neuron (*red/yellow*) with a dendrite targeting the OVLT (midline plexus of GnRH neuronal processes endogenously labeled with GFP, *green*) and exhibiting a branching, irregular dendritic tree within the OVLT. a, b, and c, Position of high-power single-plane confocal images showing the presence of spines (*arrowheads*) on the dendrites within the OVLT shown in C and D. Note that the first branching dorsal-orientated dendrite has been omitted from C but is shown in D. D, Schematic traced from confocal images in C superimposed on the GFP signal in grayscale. E, Low-power view of the neuron in C and D showing its relationship to other GnRH neurons (*black*) located in the rPOA. *Scale bars*: 50 μm (B–D), 2 μm (c), and 100 μm (E). MS, Medial septum.

nal phenotypes, none of these markers colocalized to any GnRH neuron compartment (Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

We therefore undertook other approaches to identify the nature of GnRH processes in the OVLT. First, confocal microscopy analysis revealed that a great majority of the complex GnRH neuron neurites in the OVLT exhibited spines throughout their length (Fig. 1, C and D).

Second, we undertook ultrastructural studies using silver-enhanced immunogold labeling with transmission electron microscopy. In these studies, as before (12), we combined GnRH and GFP antisera to enhance our ability to examine GnRH processes. In both animals, we observed a substantial number of GnRH/GFP-labeled processes within the OVLT. We found neurites with a loose microtubular arrangement, a lack of vesicles and synapses typical of dendritic elements (Fig. 3, A and B) as well as immunoreactive elements with packed dense core vesicles, typical of presynaptic elements seen in axons (Fig. 3C).

To further examine whether the neurites were dendrites, as suggested by anatomical data, we investigated whether they responded electrophysiologically to locally applied glutamate. Such responses would not be expected in axons, which typically do not express glutamate receptors. Individual GnRH neurons from coronal brain slices prepared from five adult male GnRH-GFP mice were recorded in the on-cell configuration, and brief puffs of 1 mM glutamate (20 msec, 10 PSI) were applied to different parts of the GnRH neuron. As illustrated in Fig. 4, it was possible to induce action potential firing in GnRH neurons when a puff of glutamate was made 40 μm but not 60 μm from the soma (Fig. 4B), indicating the effective radius of a puff. When the same locally confined puff was applied to the distant (>100 μm) dendrites of five GnRH neurons, determined by following the GFP fluorescent dendrite into the OVLT, it was similarly found to evoke firing in all neurons. In two of these GnRH neurons, glutamate was effective in activating firing when puffed at any location of the visible dendrite in the OVLT. For the other three

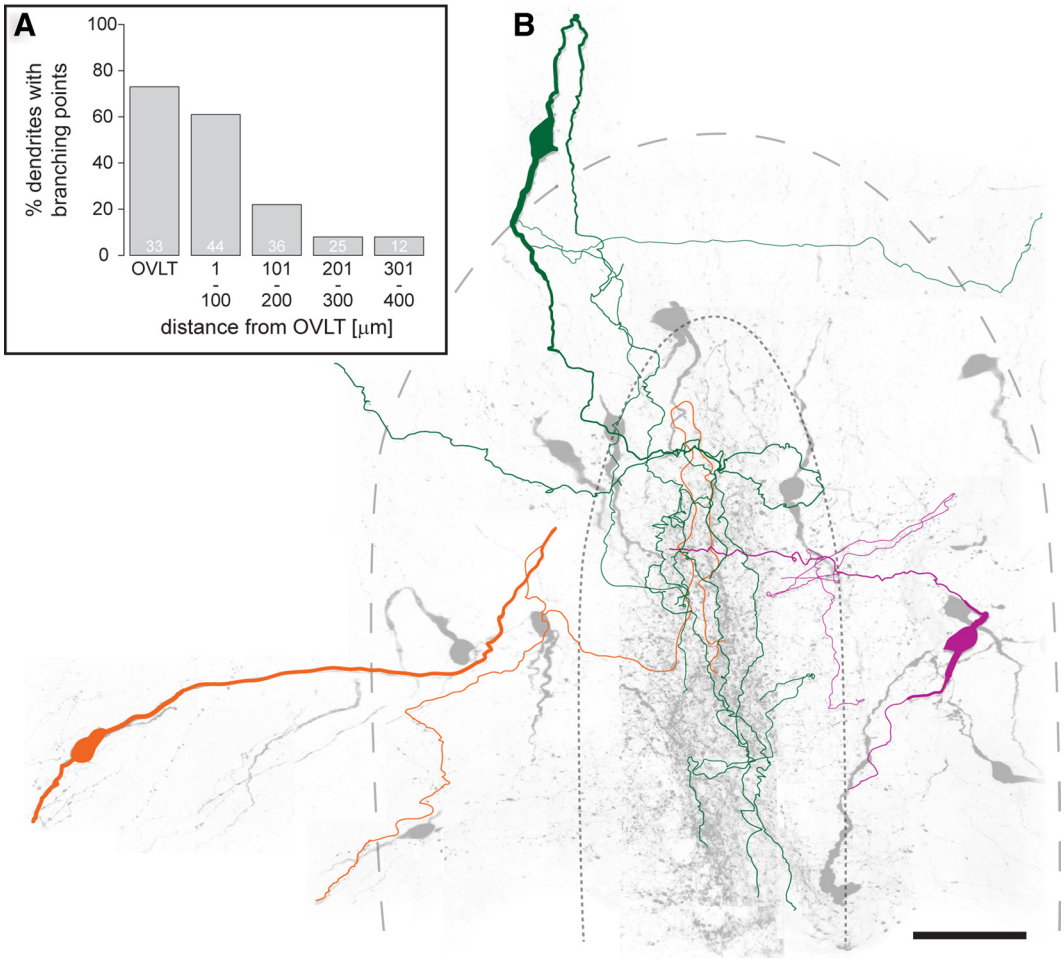

FIG. 2. Multiple GnRH neurons project complex dendritic trees into the OVLT. A, Correlation of GnRH neuronal dendritic elements exhibiting branching points to their distance from the OVLT. Distances are measured from the outer margin of the OVLT; dendritic numbers are given in white at the base of each *bar*. B, Camera-lucida-like schematic traced from confocal stack projections of three filled GnRH neurons targeting the OVLT superimposed on the endogenous GFP signal in grayscale. Note other GnRH neurons (*gray*) immediately adjacent to the OVLT. *Scale bar*, 50 μm. *Inner dashed gray line* outlines the OVLT, *outer dashed line* represents a distance of approximately 100 μm from the OVLT.

GnRH neurons, the response to glutamate was lost at more distant sites within the OVLT (Fig. 4B). Under identical recording conditions, puffs of ACSF onto cell body or dendrite did not elicit action potentials but did so after switching to the puff pipette solution with 1 mM glutamate (data not shown).

Are the complex GnRH neuron dendrites in the OVLT outside the BBB?

The results above suggested that GnRH neuron dendrites in the OVLT were outside the BBB. To examine the extent to which GnRH neuron dendrites were exposed to blood-borne molecules, we injected the enzyme HRP (molecular weight 44,000) iv into six anesthetized adult male mice. HRP is unable to cross the BBB, and iv injection has been used in several studies to assess the integrity of the BBB (23–25). Five or 25 min after injection, mice were perfused with paraformaldehyde. In all six mice, histochemical analysis showed the presence of HRP within the OVLT (Fig. 5A), median eminence, and base of the arcuate nucleus (Fig. 5B) as well as subfornical organ (data not shown). The remainder of the forebrain was completely negative for HRP staining as were the circumventricular organs of saline injected mice (Fig. 5A). Curiously, the median eminence was labeled less than the arcuate nucleus, despite its supposedly higher density of fenestrated capillaries (Fig. 5B). When dual labeled for GnRH and HRP, a marked overlap was found between the two signals in the OVLT and median eminence/arcuate nucleus (Fig. 5). We found that HRP was present in the OVLT as well as in the adjacent parenchyma for up to approximately 100 μm from the OVLT and that this involved all GnRH neuron dendrites as well as cell bodies located in this region (Fig. 5A). This indicates that all of the GnRH elements in the OVLT itself and within a approximately 100-μm radius will be exposed to molecules in the peripheral circulation.

GnRH neuron dendrites in the OVLT respond to kisspeptin

The neuropeptide kisspeptin is a potent activator of GnRH neuron firing (26) and strongly stimulates the re-

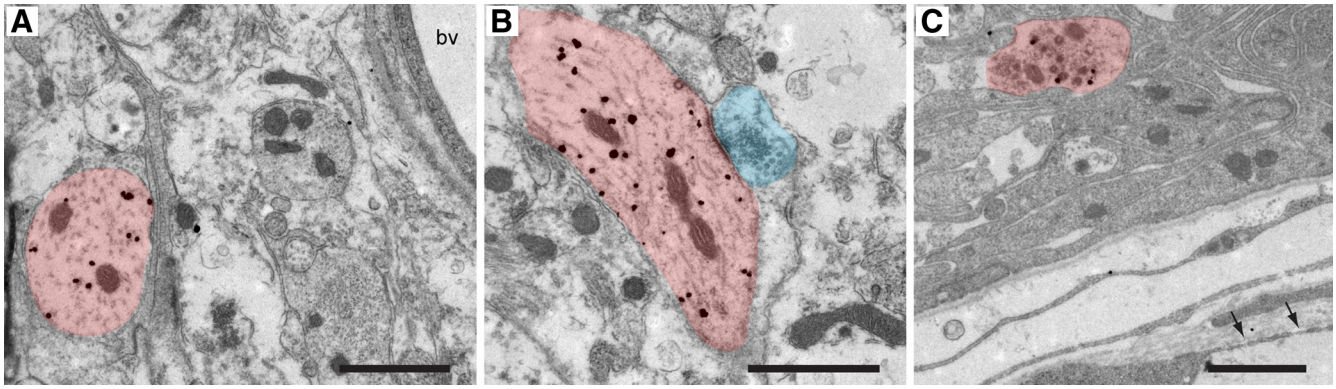

FIG. 3. Ultrastructural evidence for GnRH neuron dendritic elements in the OVLT. A, Representative GnRH neuronal element (*shaded in red*) identified by silver-enhanced immunogold labeling in the OVLT. A and B, Both elements show dendritic characteristics of loosely arranged microtubules and absence of vesicles. B, A GnRH-labeled dendrite in the OVLT targeted by a synapse (*shaded in blue*). C, GnRH neuronal element (*shaded in red*) showing axonal characteristics of densely packed microtubules and containing dense core vesicles, some of which are silver-enhanced immunogold labeled for GnRH. Fenestrations of a blood vessel in the vicinity are indicated by *arrows*. *Scale bars*, 1 μm. bv, Blood vessel.

lease of GnRH (27). Interestingly, peripherally administered kisspeptin can elicit a GnRH-dependent increase in LH secretion (27–29). The mechanism of this phenomenon is unclear, because kisspeptin cannot cross the BBB. However, GnRH neuron dendrites in the OVLT could be in a position to sense blood-borne kisspeptin. To investigate whether GnRH neuron dendrites within the OVLT can respond to kisspeptin, we applied brief puffs (5 sec, 6 PSI) of 100 nM kisspeptin to the OVLT area only. A puff of lower pressure but longer duration (compared with glutamate puffs) was chosen to maximize the strength of stimulation while maintaining the local confinement to the OVLT area. This was able to induce a moderate increase in the firing rate of 91% of GnRH neurons (n = 11, from

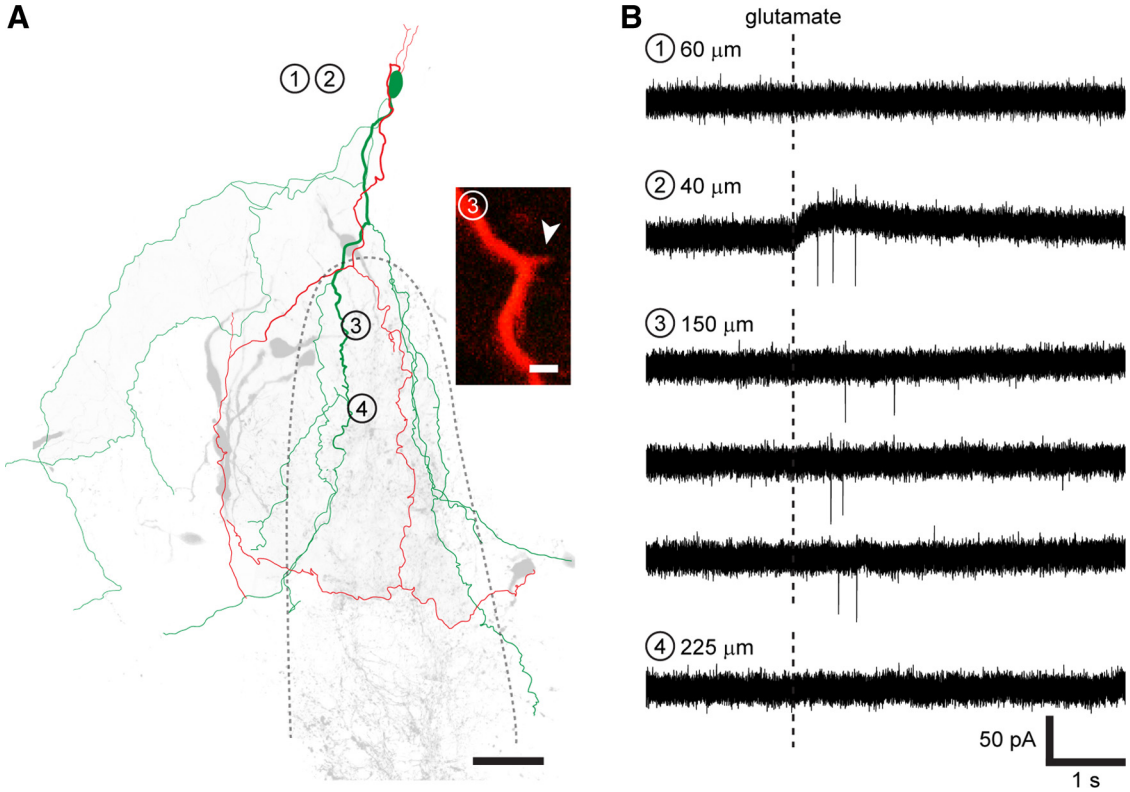

FIG. 4. GnRH neuron dendrites within the OVLT respond to glutamate. A, Camera-lucida picture of the dendritic tree of a single GnRH neuron that responded to brief puffs of 1 mM glutamate. For clarity, the dorsal and ventral dendrites of this GnRH neuron have been color-coded *red and green*, respectively. *Numbers in circles* indicate four sites of glutamate application, corresponding traces are shown in B. *Inset* shows a high-power single-plane confocal image of the dendrite at site 3 bearing a spine (*arrowhead*). B, Puffs of 20-msec duration with 1 mM glutamate did not evoke action currents in the GnRH neuron cell body when applied 60 μm from the soma (*number 1*), whereas closer applications at 40 μm evoked firing (*number 2*). The dendrite at 150 μm from the soma in the OVLT (*number 3*) responded reproducibly to glutamate, but responsiveness was lost in more central areas of the OVLT (*number 4*). *Scale bar*, 50 μm (A) and 2 μm (*inset*). *Dashed line* in A outlines the OVLT.

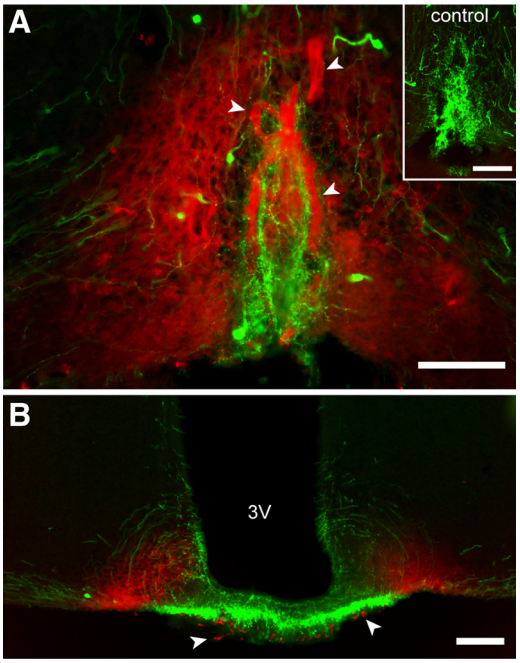

FIG. 5. Defining the extent of the BBB and diffusion from the OVLT. A, Intravenously injected HRP (*pseudocolored in red*) appears in the OVLT and also approximately 100 μm beyond its borders within the adjacent parenchyma. This includes all of the GnRH elements within the OVLT as well as cell bodies and dendrites in the vicinity (*green*). The *inset* shows the OVLT of a saline injected animal completely devoid of HRP signal. B, The median eminence/arcuate nucleus region is another confined area of HRP entry and diffusion. *Scale bars*, 100 μm. *Arrowheads* indicate labeled blood vessels. 3V, Third ventricle.

nine male and two female mice) that projected dendrites into the OVLT (Fig. 6, A and B). Five neurons (45%) were activated more than 2 SD above the baseline average. Because the elicited response and stimulus used was much weaker than previously reported, we subsequently applied 100 nM kisspeptin in the bath for 1 min to confirm the general responsiveness of the recorded neuron to kisspeptin (Fig. 6) (26). This treatment triggered a long-lasting increase in firing in 78% of the cells tested (n = 9).

Do GnRH neurons with dendrites in the OVLT participate in the GnRH/LH surge?

The induction of c-Fos is considered to be a good indicator of which GnRH neurons are involved in the GnRH/LH surge mechanism (30). To examine whether GnRH neurons with OVLT-directed dendrites might be involved in this mechanism, we performed dual-labeling for GnRH and c-Fos on rPOA brain sections from five ovariectomized, steroid-primed female mice that had exhibited an LH surge (8.8 ± 1.3 ng/ml LH). We then analyzed their brains focusing upon those GnRH neurons located within 100 μm of the OVLT, because approximately 70% of these cells give rise to dendrites that project into the OVLT (Fig. 2A). Dual-labeled cells exhibited brown cytoplasmic staining for GnRH and black nuclear-located labeling for c-Fos (Fig. 7A) exactly as reported previously (30). Dual-labeled GnRH neurons were identified throughout the rPOA but concentrated around the OVLT (Fig. 7B). Although 66 ± 4% of GnRH neurons located throughout the POA expressed c-Fos, 86 ± 5% of GnRH neuron cell bodies located with 100 μm of the OVLT were positive for c-Fos.

Discussion

We provide here evidence that adult GnRH neurons do not necessarily have a simple bipolar morphology but, rather, can exhibit complex, highly branched dendritic trees. We also demonstrate that some GnRH processes in the OVLT are dendritic in nature rather than axonal as has been assumed previously. Most GnRH neurons with cell bodies or dendrites located within approximately 100 μm of the OVLT were found to extend highly complex dendritic trees into the OVLT. We estimate this subpopulation of GnRH neurons to represent more than 20% of all GnRH neurons located in the rPOA. Further, we show that many of the GnRH-immunoreactive processes described in the OVLT are in fact dendrites and lie outside the BBB.

The simple bipolar morphology of the GnRH neuron has been recognized for several decades (6). The recent ability to fill preidentified GnRH neurons *in situ* with small molecular weight dyes has lead to the observation that these bipolar GnRH neurons have remarkably long, typically unbranched dendrites often extending over 1000 μm (10, 12), a rather unique dendritic morphology within the mammalian nervous system. We now report the surprising observation that a subpopulation of rPOA GnRH neurons exhibits an incredibly complex dendritic tree with multiple branches and irregular organization. Unlike other features of GnRH neurons that are rather heterogeneous within the population (8), GnRH neurons with complex dendrites show a tight topographical clustering around the OVLT. Indeed, the complex dendritic trees of these GnRH neurons appear to target the OVLT. This relationship to the OVLT was emphasized by the repeated observation of bipolar GnRH neurons extending simple unbranched dendrites until nearing the OVLT whereupon they start branching and send multiple processes into this area. We show that this apparent "branch inductive zone" exists within a 100-μm radius around the OVLT. This region is strikingly similar to the zone into which HRP was able to penetrate and raises the intriguing possibility that blood-borne factors may have a role in inducing and/or maintaining this extensive dendritic branching in the OVLT region.

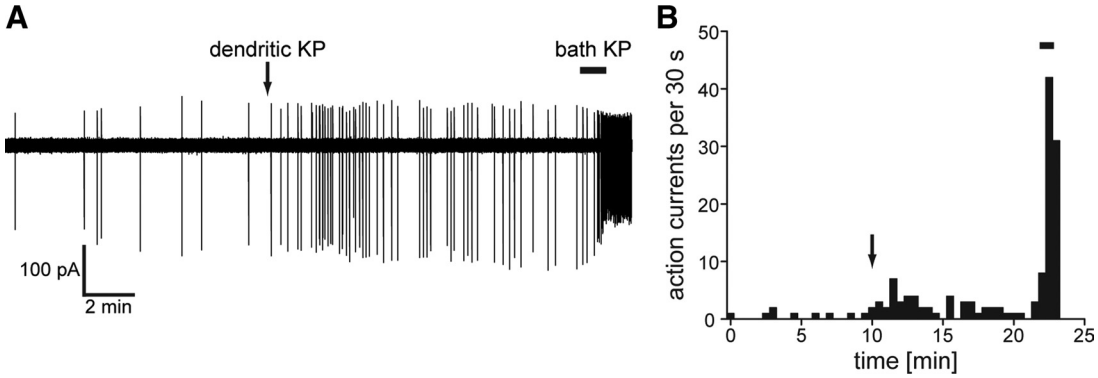

FIG. 6. GnRH neuron dendrites within the OVLT respond to kisspeptin. A, Trace of a GnRH neuron with dendrites in the OVLT during 5-sec puff of 100 nM kisspeptin in the OVLT (*arrow*) and bath application of 100 nM kisspeptin (KP, *bar*). The trace was high-pass filtered at 2 Hz to remove baseline fluctuations. B, Histogram showing the number of recorded action currents in A in 30-sec bins. Applications of kisspeptin are indicated as in A.

The dense GnRH fiber plexus of the OVLT is a well-described feature of the GnRH neuronal system. On the basis of immunocytochemical studies, it has been assumed that this represented GnRH axons (31–33). To our knowledge, only one ultrastructural study of GnRH elements in the OVLT has been undertaken, and this reported the presence of immunoreactive string-like varicosities interpreted as axons (34). Using a GnRH+GFP immunocytochemical labeling protocol, which enhances our ability to detect GnRH neuron elements in ultrastructural studies (12), we find here that GnRH/GFP-immunoreactive elements in the OVLT of the GnRH-GFP mouse can exhibit axonal or dendritic morphologies. Thus, as indicated by the ultrastructural studies, and more quantitatively by the confocal studies, many of the GnRH processes in the OVLT are in fact dendrites and not axons. In contrast to what has been reported for axon terminals in the median eminence (7), GnRH-immunoreactive elements in the OVLT were not found here to be associated with blood vessels (34). We note, however, that a comprehensive ultrastructural evaluation of the OVLT in different animal groups needs to be undertaken to confirm this observation.

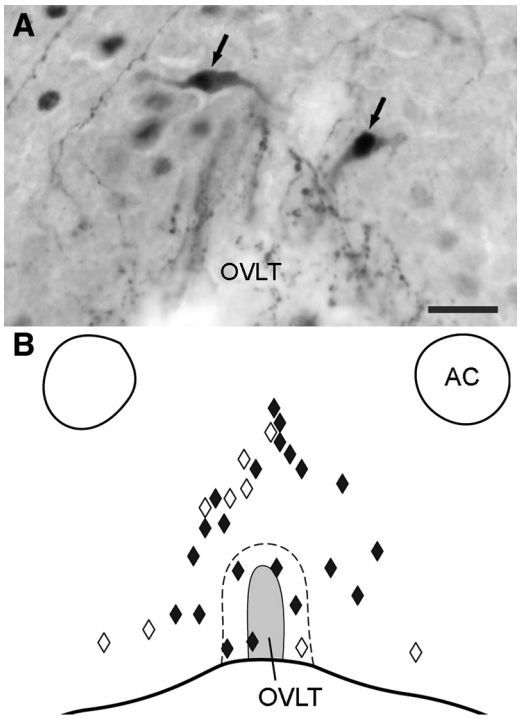

FIG. 7. Activation of GnRH neurons around the OVLT during the GnRH/LH surge. A, The image of a chromagen double label for GnRH (cytoplasmic) and c-Fos (nuclear) in neurons (*black arrows*) located in the dorsal cap of the OVLT within the 100-μm zone at the time of the GnRH/LH surge. B, Schematic of a representative coronal section showing the locations of rPOA GnRH neurons with (*filled diamond*) and without (*unfilled diamond*) nuclear c-Fos labeling. The OVLT is *shaded in gray*, and a 100-μm radius zone is indicated with a *dashed line*. *Scale bar*, 20 μm. AC, Anterior commissure.

We have used the presence of spines, ultrastructural analysis, and electrophysiology to show that the complex neurites in the OVLT are dendritic in nature. However, it remains curious that a wide array of classical markers of different neuronal compartments has been negative in GnRH neurons. This may result from their unusual origin from the nasal placode (35) and suggests that structural elements within GnRH neurons may be different from other neuronal phenotypes.

The function of GnRH neuron dendrites in the OVLT is not established as yet but can be inferred, in part, from their morphology and location. We find evidence for multiple spines, synapses, and fast responses to glutamate and kisspeptin demonstrating that GnRH neuron dendrites in the OVLT are regulated by classical and peptidergic transmitters in the normal manner. However, the unique aspect of these complex dendritic trees is their presence outside the BBB. As described previously (23), we show that large molecular weight, blood-borne molecules are able to pass through the capillaries of the OVLT and even disperse out into the adjacent parenchyma for distances up to 100 μm. This indicates that, in addition to dendrites within the OVLT, the more medially located GnRH neuron dendrites and cell bodies in the rPOA will also very likely be exposed to the constituents of peripheral blood. Thus, GnRH neuronal elements in and

around the OVLT will not only be regulated in the normal manner by neurotransmitter inputs but also be able to monitor directly molecules circulating in the blood.

Of particular interest, we show here that kisspeptin does not need to cross the BBB to activate GnRH neurons as GnRH neuron dendrites in the OVLT respond to kisspeptin. As would be expected, the activation of GnRH neuron firing observed after small puffs of kisspeptin onto their dendrites in the OVLT was less potent than full activation of the GnRH neuron by bath application of kisspeptin. This observation may help explain the finding that kisspeptin given into the periphery is able to activate LH secretion despite its likely inability to cross the BBB (27–29). Alongside the proposed action of kisspeptin at GnRH nerve terminals in the median eminence (36, 37), this work indicates that kisspeptin in the peripheral blood can also act on GnRH neuron dendrites in the OVLT to stimulate LH secretion.

To provide some initial information about the roles of GnRH neurons with dendrites in the OVLT, we examined whether they might be involved in the surge mechanism. We find here that approximately 85% of GnRH neurons with cell bodies located within 100 μm of the OVLT express c-Fos at the time of the GnRH/LH surge. Because approximately 70% of GnRH neurons in this area extend dendrites into the OVLT, it seems reasonable to conclude that the majority of GnRH neurons with dendrites in the OVLT is activated at the time of the GnRH/LH surge and likely contributes to the GnRH surge at the median eminence. Thus, many GnRH neurons contributing to the GnRH/LH surge have their dendritic tree exposed to blood-borne constituents.

The OVLT is well established as a key site in the regulation of body fluid homeostasis with OVLT neurons responding directly to changes in blood osmolarity and angiotensin (2, 3, 38). Neurons and glial cells within the OVLT are also known to respond to cytokines such as IL-6 and TNF-α and play a role in the febrile response to inflammatory processes and stress (39–42). Although the impact of disordered fluid homeostasis on GnRH neurons is not known, it is evident that inflammatory cytokines act to suppress the reproductive axis (43, 44). Mouse GnRH neurons express a wide array of cytokine receptors (45), and we now report here that the dendrites of these cells are located in an established circumventricular organ known to monitor directly circulating inflammatory cytokine levels. Previous investigations of circumventricular organs have concentrated upon defining the effects of various compounds on the electrical or biosynthetic activity of neural and glial cell bodies within these structures (3). The active targeting of dendrites into brain regions lacking a BBB, as exemplified here for GnRH neurons, represents a previously overlooked mechanism through which the periphery would be able to modulate a much larger number of neurons than previously suspected.

In summary, we report here that a subpopulation of GnRH neurons in the rPOA has a much more complex dendritic morphology than previously described and that they direct highly branched, irregular dendritic trees into the OVLT. These dendrites arise from GnRH neurons involved in the GnRH/LH surge mechanism and extend outside the BBB, where they are able to directly sense blood-borne molecules. This indicates a new mechanism for the modulation of GnRH neurons that extends considerably the range of factors that are integrated by these neurons for the control of fertility.

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