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The Prairie Vole Vomeronasal Organ is a Target for Gonadotropin-releasing Hormone

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Abstract

Gonadotropin-releasing hormone (GnRH) is present in nervus terminalis neurons in chemosensory nerve fascicles in vertebrates. In rodents, the majority of GnRH fibers are located within vomeronasal nerves. We have shown that GnRH can alter vomeronasal receptor neuron responses to odors. In this study, using prairie voles, we tested the hypotheses that (i) GnRH-immunoreactive (-ir) neurons project to the vomeronasal organ and accessory olfactory bulb; (ii) a radioactive-labeled GnRH agonist, buserelin, binds to vomeronasal sensory neurons; and (iii) vomeronasal receptor cells express GnRH receptor mRNA as evidenced by reverse transcription–polymerase chain reaction (RT–PCR) combined with Southern blotting. In neonatal voles, GnRH-ir cell bodies and fibers were observed within the vomeronasal epithelium, vomeronasal nerves and accessory olfactory bulbs. In adult voles, GnRH-ir fibers were observed not only in the lamina propria of the vomeronasal mucosa, but also along vomeronasal nerves and in the accessory olfactory bulb. Binding of [^{125}I]buserelin was observed specifically over the vomeronasal sensory epithelium, and RT–PCR/Southern blotting demonstrated GnRH receptor expression in the vomeronasal mucosa, as well as in olfactory epithelium and pterygopalatine ganglion, two additional structures containing GnRH-ir neurons of the nervus terminalis. This study supports the hypothesis that GnRH is released from nervus terminalis fibers to modulate chemosensory processes, especially those involving chemoreception in the vomeronasal organ.

Introduction

Gonadotropin-releasing hormone (GnRH) is a neurohormone involved in a number of reproductive processes, including release of gonadotropin hormones from the pituitary gland and stimulation of brain centers that initiate or facilitate sexual behavior. The neurons that synthesize and release GnRH are present within various regions of the forebrain, midbrain and several peripheral neural structures related to olfaction, such as nasal mucosa (Schwanzel-Fukuda and Silverman, 1980) and cranial autonomic ganglia (Wirsig-Wiechmann and Lepri, 1991; Wirsig-Wiechmann, 1993). Within the rostral forebrain and the peripheral olfactory structures, the GnRH neurons are considered to be part of the nervus terminalis system (Figure 1). This neural system is present in all vertebrates and is thought to act as a neuromodulatory system for both peripheral (Eisthen *et al*., 2000; Wirsig-Wiechmann *et al*., 2000) and central brain regions (Oka, 1992).

GnRH receptors have been visualized by *in vitro* autoradiography (Badr and Pelletier, 1987; Jennes *et al*., 1988; Jennes and Conn, 1994) and *in situ* hybridization (Jennes *et al*., 1997) in rodent brain, demonstrating the sites of central GnRH action. The presence of GnRH in peripheral chemosensory-related structures suggests that GnRH modulates chemoreception at the level of transduction of chemosensory signals or modulates autonomic inputs to chemosensory structures. We have previously shown that a GnRH agonist, buserelin, binds to tiger salamander olfactory neurons (Wirsig-Wiechmann and Jennes, 1993) and that GnRH modulates olfactory neuron voltage-gated responses in mudpuppies (Eisthen *et al*., 2000). Our recent findings indicate that GnRH can specifically modulate olfactory and vomeronasal neuron responses to odors in rodents (Wirsig-Wiechmann *et al*., 2000). In rodents, the majority of peripheral nervus terminalis fibers projects to the vomeronasal organ, suggesting that the main focus of GnRH action in the periphery is to control vomeronasal detection of chemosensory stimuli.

In the present study, we used prairie voles to visualize the projections of the peripheral nervus terminalis and determine whether a GnRH agonist binds to vomeronasal neurons and whether vomeronasal mucosa expresses the GnRH receptor. We used prairie voles because their nervus

terminalis system demonstrates the most robust labeling in immunocytochemical studies designed to study the projections of the GnRH-immunoreactive (-ir) neurons in the nasal cavity. We have confirmed that GnRH-ir neurons of the nervus terminalis project directly to the vomeronasal organ, that binding of the GnRH agonist, buserelin, occurs specifically to vomeronasal chemosensory neurons and that the vomeronasal cells express the GnRH receptor. These findings support our previous data that GnRH influences the processing of chemical stimuli at the sensory receptor level (Eisthen *et al*., 2000; Wirsig-Wiechmann *et al*., 2000).

Materials and methods

Animals

Ten neonatal and six adult prairie voles were used in this study for immunocytochemical analysis of GnRH projections in the nasal cavity. Two adult voles were used for *in vitro* GnRH agonist binding (Wirsig-Wiechmann and Jennes, 1993) and two adult male voles were used for determination of GnRH receptor expression by reverse transcription–polymerase chain reaction (RT–PCR) and Southern blotting. Voles were obtained from breeding colonies in the laboratories of Dr John Lepri (University of North Carolina, Greensboro, NC) and Dr Thomas Insel (Emory University, Atlanta, GA). Animals were maintained on a diet of rabbit chow and sunflower seeds and were housed in plastic cages with wood chips. Animals were kept on a 14 h light/10 h dark illumination schedule. The animals were cared for in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*.

Immunocytochemistry

Voles were anesthetized by halothane inhalation and perfused transcardially with saline followed by Zamboni's fixative (2% paraformaldehyde–15% picric acid in 0.1 M sodium phosphate buffer, pH 7.4). Adult heads were decalcified in DeCal (Omega Chemical Corp., Cold Spring, NY) and all tissues were cryoprotected in 30% sucrose-phosphate buffer. Tissue was sectioned (20 μm) in the sagittal plane on a cryostat microtome. Tissue was placed directly onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA), air-dried and stored at –80°C until immunocytochemical processing.

For immunocytochemistry, tissue was rinsed in six 5 min rinses of phosphate buffered saline (PBS), pH 7.4, between incubations. Tissue was incubated in rabbit anti-GnRH antisera (1:4000; Lot No. 635479; DiaSorin, Stillwater, MN) or guinea pig anti-GnRH antisera 6–4 (1:4000; kindly supplied by Dr Lothar Jennes) for 3 days at 4°C, followed by a 30 min incubation in each of the following: biotinylated goat anti-rabbit IgG (or biotinylated goat anti-guinea pig IgG) and avidin-conjugated horseradish peroxidase (Vector Laboratories, Burlingame, CA). Labeling was conducted with either diaminobenzidine (DAB) or Chromo-red (3-amino-9-ethylcarbazole; Sigma, St Louis, MO). For DAB processing, tissue was rinsed in PBS and then immersed in 0.05 M Tris, pH 7.4, for 5 min, then in 0.05% DAB, 0.001% hydrogen peroxide in 0.05 M Tris buffer, pH 7.4, for 10 min and finally rinsed in PBS. Tissue labeled with DAB was counterstained with methyl green, dehydrated and coverslipped with Permount. For Chromo-red labeling, tissue was rinsed in PBS, followed by a 5 min incubation in 0.01 M acetate buffer, pH 5.2, and a 20 min incubation in 0.02% 3-amino-9-ethylcarbazole, 5.55% *N*,*N*-dimethylformamide, 0.33% hydrogen peroxide in 0.01 M acetate buffer, pH 5.2. Following a rinse in PBS, tissue labeled with Chromo-red was coverslipped with glycerol gelatin (Sigma). For immunocytochemical control conditions, tissue was incubated in GnRH antisera preabsorbed overnight with 20 μM synthetic mammalian GnRH, or in preincubation solution containing no primary antisera. These incubations were followed by normal labeling procedures. Tissue was observed with an Olympus microscope (Melville, NY) and digital images were acquired with a SPOT camera (Diagnostic Instruments, Sterling Heights, MI).

Receptor binding autoradiography

Voles were anesthetized by halothane inhalation and killed by decapitation. Vomeronasal organs were quickly removed and the mucosa was dissected from the bone, frozen in liquid nitrogen and fresh-frozen sectioned (30 μm) coronally with a cryostat microtome. Tissue sections were placed directly onto three sets of Superfrost Plus slides (Fisher) and two sets were preincubated in 5 mM Tris–HCI, pH 7.4, containing 0.2% bovine serum albumin at 4°C for 30 min. Tissue was then incubated in the same buffer containing [^{125}I]buserelin (200 000 c.p.m./ml; 1 mCi/g) with or without unlabeled GnRH (25 μM) for 2 h at 4°C. Following four 1 min buffer rinses and two 30 s water rinses, tissue was dried under a cold stream of air and exposed to P-Max Hyperfilm (Amersham Pharmacia, Piscataway, NJ). Experimental and control tissues were processed together and exposed to the same Hyperfilm to allow direct comparisons. Following a 3 day exposure, autoradiographs were developed and slides for which binding was apparent were vapor-fixed with paraformaldehyde and dipped in autoradiographic emulsion (Ilford NTB-2). Slides were developed after a 1 month exposure. The third set of tissue was fixed with paraformaldehyde and stained immediately with cresyl violet. Because buserelin binding requires the use of unfixed tissue, the morphology of tissue is not optimal for visualization of specific cells. However, since the sensory epithelium of the vomeronasal organ is very well localized to the medial wall of the organ and since the sensory cells form a thick epithelium, a regional analysis of the binding of buserelin can be readily accomplished.

For visualization of relative silver grain densities generated from buserelin binding, computer-generated color

images were obtained from film autoradiographs. Autoradiographic film images were digitized with 241 density levels. Six colors were chosen for ranges of silver grain densities. For numerical silver grain density measurements, 10 selected areas from all experimental and adjacent competition control vomeronasal tissues sections were analysed using NIH image software. Density values were taken from randomly chosen vomeronasal sensory cell areas of varying size and location. The possible numerical value range extended from 1 (no silver grains, i.e. white) to 241 (completely filled with silver grains, i.e. black). Student's *t*-test (two-tail) was used to compare the densities between the various regions (unpaired values).

RT–PCR

Two male voles were killed by carbon dioxide inhalation and decapitation. Vomeronasal mucosa, olfactory epithelium and pterygopalatine ganglia were removed bilaterally and each tissue type was pooled and frozen separately in liquid nitrogen. Tissue was stored at –80°C until use. For RNA isolation, tissue was homogenized and RNA extracted using an RNeasy Total RNA Isolation Kit (Qiagen, Santa Clarita, CA), according to the manufacturer's instructions. Extracted RNA was further treated with DNase to remove all genomic DNA, using a Message Clean Kit (GenHunter Corp., Nashville, TN). Total RNA (1 μg/sample) was reverse transcribed (RT) to cDNA with the use of a RNA PCR kit (Perkin-Elmer, Norwalk, CT) in a total volume of 10 μl. The cDNA was then amplified by PCR using primers complementary to the cloned rat GnRH receptor sequence to yield a 257 bp fragment. The 5′ primer sequence was [5′-TGA TTA GCC TGG ATC GCT CC-3′] and the 3′ primer sequence was [5′-GAA CAG GCA GCT GAA GGT-3′], which correspond to positions 401–420 and 657–638, respectively, and were synthesized by Integrated DNA Technologies Inc. (Coralville, IA). These primers were complementary to sequences on exons 1 and 2 of the rat GnRH receptor gene. In addition, both primers encoded portions of the third and fifth transmembrane regions of the receptor and therefore are highly conserved across species. The 5′ primer sequence (positions 401–420) and the 3′ primer sequence (positions 657–638) are both 90% homologous to the mouse sequence and the amplified region (positions 401–657) is 89% homologous to the mouse sequence (GenBank Accession No. L001119). In addition, since one intron is located between the two exons from which primers were designed, amplification of any possible contaminating genomic DNA would not result in a PCR product of the predicted size of 257 bp. PCR amplification was conducted with one cycle of 94°C for 4 min and then 35 cycles of 94°C for 30 s, 50°C for 1 min and 72°C for 1 min, followed by one cycle of 72°C for 7 min. The amplified cDNA (15 μl) was then electrophoresed on an agarose gel and stained with ethidium bromide.

Southern blot analysis

Full-length ^{32}P-labeled GnRH receptor cDNA was synthesized from a Bluescript II plasmid containing the rat GnRH receptor insert, obtained courtesy of Dr William Chin (Kaiser *et al.*, 1992). The plasmid was excised with BamHI (Promega, Madison, WI) and isolated by agarose gel electrophoresis and extracted using a Qiaex II Gel Extraction Kit (Qiagen, Santa Clarita, CA). The cDNA was end-labeled with [g-^{32}P]dCTP (3000 Ci/mmol; ICN Pharmaceuticals, Inc., Costa Mesa, CA), using a Multiprime DNA Labeling Kit (Amersham Pharmacia, Piscataway, NJ) according to the manufacturer's instructions. The labeled probe was purified on a Sephadex G-50 Quick Spin column (Boehringer Mannheim, Indianapolis, IN).

PCR products electrophoresed on an agarose gel were transferred to a Zetabind nylon membrane (Cuno Laboratory Products, Meriden, CT). The cDNA immobilized on the membrane was denatured and the membrane was incubated overnight with the ^{32}P-labeled GnRH receptor cDNA probe at 50°C. The membranes were washed under high-stringency conditions and the hybridized probe was visualized by film autoradiography.

Results

Immunocytochemistry

Immunohistochemical control conditions did not produce labeling of any neurons. Following incubations in GnRH antisera, neural cell bodies and fibers containing GnRH-ir were observed along the entire vomeronasal system projection to the accessory olfactory bulb in both neonatal and adult voles (Figure 1). The GnRH-ir was confined to bipolar and unipolar neurons of the nervus terminalis system.

In neonatal vole tissue, GnRH-ir cell bodies and fibers were observed within: (1) vomeronasal organ sensory epithelium (Figure 2A,B,D); (2) vomeronasal nerve fascicles within the vomeronasal organ (Figure 2C); and (3) vomeronasal nerve fascicles projecting through the lamina propria of the nasal septum (Figure 2E) and vomeronasal nerve fascicles traveling along the medial surface of the olfactory bulb (Figure 4A) and projecting into the accessory olfactory bulb. A plexus of GnRH-ir cell bodies and fibers also existed along the medial surface of the olfactory bulb ventral to the vomeronasal nerve projection to the accessory olfactory bulb (Figure 4A). Fibers from this plexus wrapped around the olfactory peduncle. The accessory olfactory bulb received a dense innervation of GnRH-ir fibers (Figure 2F).

In adult vole tissue, GnRH-ir cell bodies and fibers were observed within vomeronasal nerve fascicles projecting along the nasal septum (Figure 3A) and along the medial surface of the olfactory bulb (Figure 4B). As in the neonatal vole, a plexus of cell bodies and fibers also existed along the medial surface of the olfactory bulb ventral to the vomeronasal nerve projection to the accessory olfactory

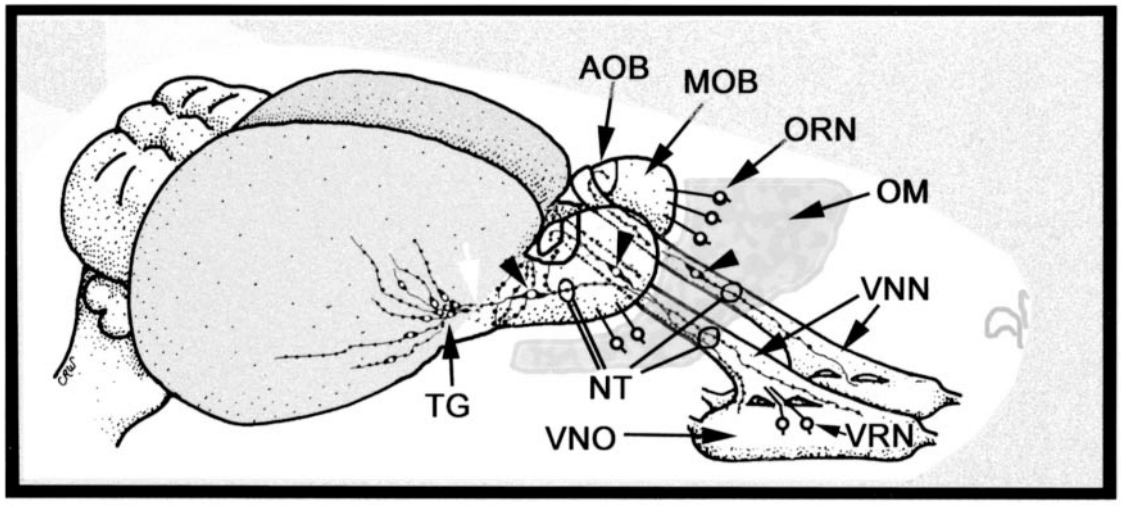

Figure 1 Diagrammatic rostrolateral view of vole brain and nasal cavity showing the location of olfactory, vomeronasal and nervus terminalis systems. The nervus terminalis (NT) is a chain of bipolar cell bodies (arrowheads) with varicose processes extending from the brain and to the vomeronasal organ (VNO). The gray region of forebrain indicates the region in which the cell bodies and processes of the nervus terminalis are located within the brain tissue. Just central to entrance of the nervus terminalis into the brain (white arrow) is a group of nervus terminalis neurons composing the terminal ganglion (TG). In the region of the main olfactory bulb (MOB), the nervus terminalis (double-line indicator) lies on the medial surface of the bulbs. In the nasal cavity the nervus terminalis (single-line indicator) is embedded within the vomeronasal nerves (VNN). The gray mottled region rostral to the MOB represents the olfactory mucosa (OM) on the nasal septum. VNN travel within the lamina propria of the septal mucosa deep to the olfactory epithelium. Two vomeronasal receptor neurons (VRN) are shown in the VNO with axons (entire trajectory not shown) projecting into the vomeronasal nerve. Abbreviations: AOB, accessory olfactory bulb; MOB, main olfactory bulb; NT, nervus terminalis; OM, olfactory mucosa; ORN, olfactory receptor neuron; TG, terminal ganglion; VNN, vomeronasal nerve; VNO, vomeronasal organ; VRN, vomeronasal receptor neurons.

bulb (Figure 4B). Some GnRH-ir fibers followed the vomeronasal nerve into the accessory olfactory bulb to provide a dense innervation of mainly the superficial layers of the accessory olfactory bulb (Figure 3B).

In both neonatal animals and adults, the GnRH-ir nervus terminalis courses over the medial surface of the olfactory bulbs (Figure 4), before entering the ventral forebrain on the medial surface of the anterior olfactory nucleus, just rostral to the anterior cerebral artery. Upon entering the forebrain, the peripheral fibers of the nervus terminalis project into a cluster of GnRH-ir neurons, the terminal ganglion (Figure 1). Caudal to the terminal ganglion, isolated GnRH-ir cells and their fibers project toward the septum and hypothalamus. On the medial surface of the olfactory bulbs, some fibers diverge from the main branch of the nervus terminalis and project to the accessory olfactory bulb within the vomeronasal nerve. In addition, other fibers project to the neck of the olfactory bulbs and completely encircle the neck of the olfactory bulb on its medial and lateral surfaces (Figure 1).

Receptor binding autoradiography

Visual examination of film autoradiographs, computer-generated color images and tissue autoradiographs demonstrated robust binding of [^{125}I]buserelin greater than that of control levels—i.e. incubation in [^{125}I]buserelin with excess non-radioactive GnRH—in the vomeronasal sensory epithelium (Figure 5). Microscopic examination of tissue autoradiographs showed specific binding in sensory cell bodies of the vomeronasal epithelium (Figure 5D).

Densitometric analysis of tissue autoradiographs revealed a higher density of silver grains (i.e. 2.5 times higher than non-specific density) in the vomeronasal sensory epithelium following incubation in [^{125}I]buserelin than in control incubations. The average density of silver grains in tissue incubated with [^{125}I]buserelin (75.7 densitometry units; 31.4% of maximum density) was significantly greater than for control incubations (32.6 densitometry units; 13.5% of maximum density). Statistical analysis showed that this difference was significant ($t = 13.43$, d.f. = 18, $P < 0.0000000001$).

RT–PCR and Southern blotting

All PCR bands demonstrated positive labeling by Southern blot analysis (Figure 6). As expected, the vomeronasal mucosa revealed the highest degree of labeling by Southern blotting. The pterygopalatine ganglion demonstrated the least PCR product as indicated by Southern blotting. This may have been due to the small amount of GnRH-receptor-expressing cells in the tissue sample.

We have previously PCR-amplified GnRH receptor mRNA from rat vomeronasal mucosa, olfactory epithelium and several other tissues with these primers and have found positive labeling by Southern blot analysis using the same

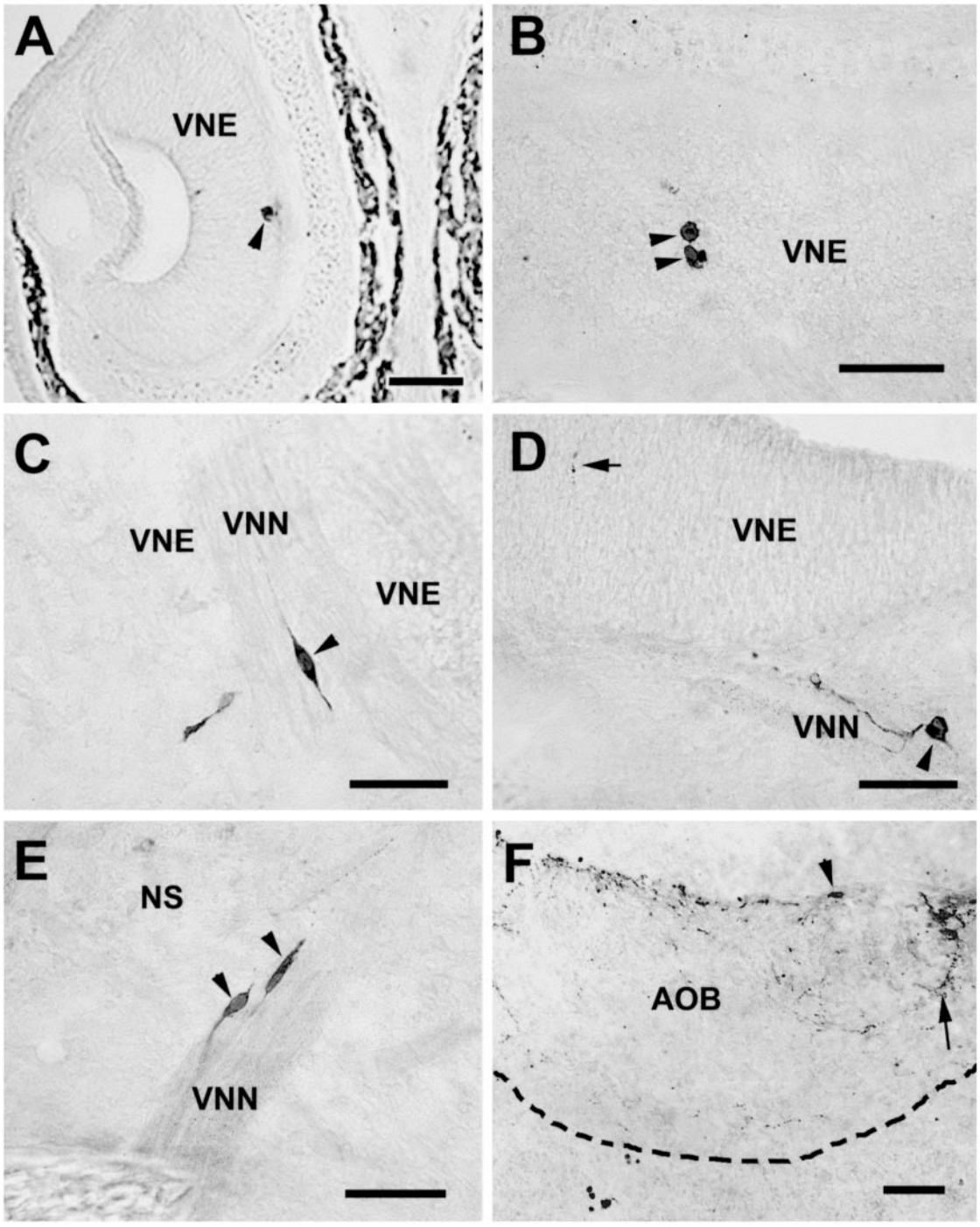

Figure 2 Micrographs of GnRH-ir neurons associated with vomeronasal system structures in the postnatal-2-day vole. **(A)** Coronal section of VNO showing a GnRH neuronal cell body (arrow head) in the basal layer of vomeronasal epithelium (VNE). **(B, C)** Sagittal section of VNO showing GnRH neuronal cell bodies within the vomeronasal bony capsule (arrow heads) in association with the VNE or vomeronasal nerve (VNN) fascicles leaving the organ. **(D)** Sagittal section of VNO demonstrating the presence of GnRH fibers (arrow) within the VNE. **(E)** Sagittal section of nasal septum showing GnRH neurons (arrow heads) within VNN fascicles projecting centrally. **(F)** Sagittal section through the accessory olfactory bulb (AOB) showing the projection of GnRH fibers (arrow) into the AOB accompanied by occasional cell bodies (arrow head). Bars = 50 μm.

full-length ^{32}P-labeled GnRH receptor cDNA (Wirsig-Wiechmann *et al*., 2000). In addition, we cloned PCR bands from rat vomeronasal mucosa and olfactory epithelium into pCRII vectors and sequenced these to confirm the identity of the amplified cDNAs. The nucleotide sequences from the cloned cDNAs demonstrated 100% bp identity with the published sequence for the mammalian GnRH receptor (GenBank Accession No. L07646).

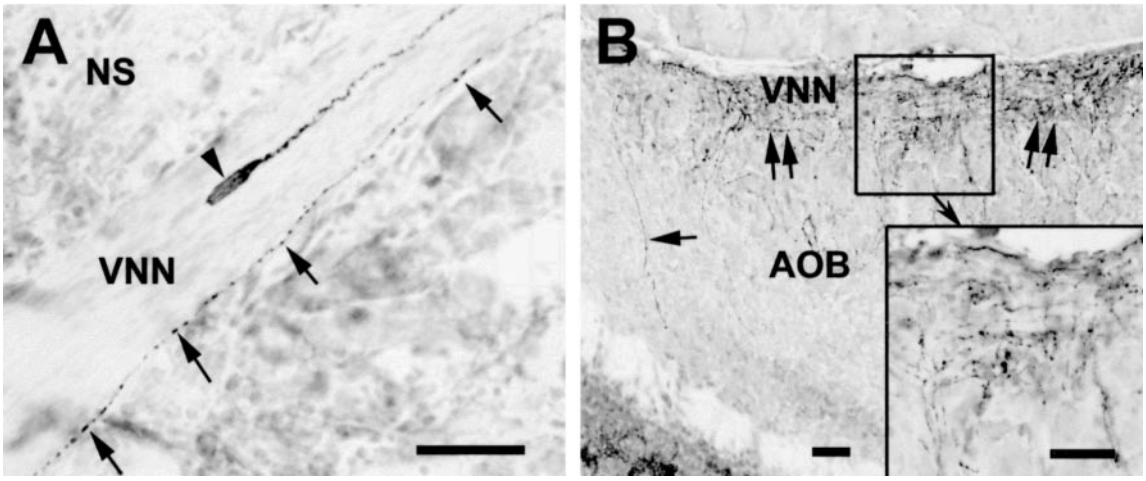

Figure 3 Micrographs of GnRH-immunoreactive neurons associated with vomeronasal system structures in the adult vole. **(A)** Sagittal section of nasal septum showing GnRH neuron cell bodies (arrow head) and varicose fibers (arrows) within vomeronasal nerve (VNN) fascicles. **(B)** Sagittal section through the accessory olfactory bulb (AOB) showing the projection of GnRH fibers (double arrows) into the AOB with some fibers projecting to the deep layers of the AOB (single arrow).Inset: magnification from boxed region at the dorsal surface of the AOB showing that fibers are numerous and varicose. Bars = 50 μm.

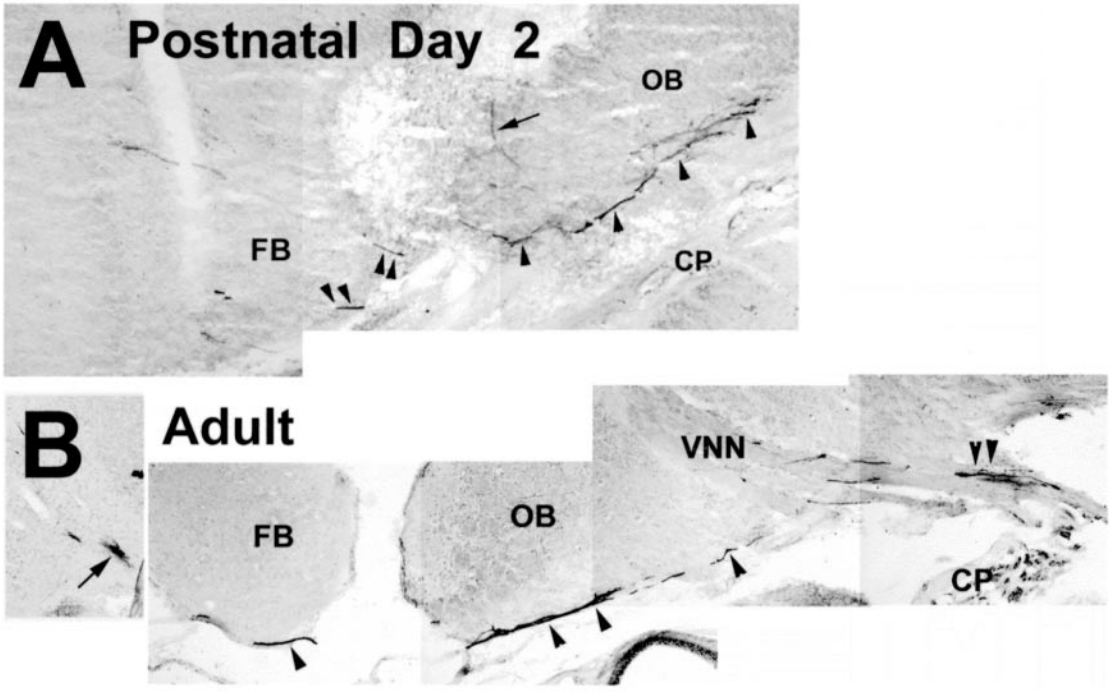

Figure 4 Micrograph-montages of sagittal sections through the medial surface of olfactory bulbs (OB) and forebrain (FB) of **(A)** postnatal-day-2 and **(B)** adult voles. Micrographs show the trajectory of the GnRH-immunoreactive nervus terminalis (arrow heads). The nervus terminalis travels along the ventromedial surface of the main OB and enters the FB in the region of the posterior part of the anterior olfactory nucleus (double arrow heads in A; single arrow in B). Some fibers separate from the main nervus terminalis bundle and travel along with the vomeronasal nerves (VNN in B) to the accessory OB (not show in micrographs since it is more laterally placed).The VNNs are not clearly defined in the neonatal tissue (A), but the GnRH-ir projection is visible (arrow). The GnRH projection along the OBs is continuous with the projection within VNNs in the nasal cavity. This projection (double arrowheads in B) can be seen within VNNs entering the cranium through the cribriform plate (CP). Bar = 200 μm.

Discussion

This study has demonstrated that GnRH-ir fibers project directly to the vomeronasal organ in prairie voles. It also supports our hypothesis that GnRH binds specifically to vomeronasal sensory epithelium and that vomeronasal mucosa expresses the GnRH receptor. GnRH-ir neural cell bodies and fibers were mainly associated with the vomeronasal systems in both neonatal and adult voles. Very little GnRH-ir was observed in association with olfactory structures at both peripheral and central levels. These

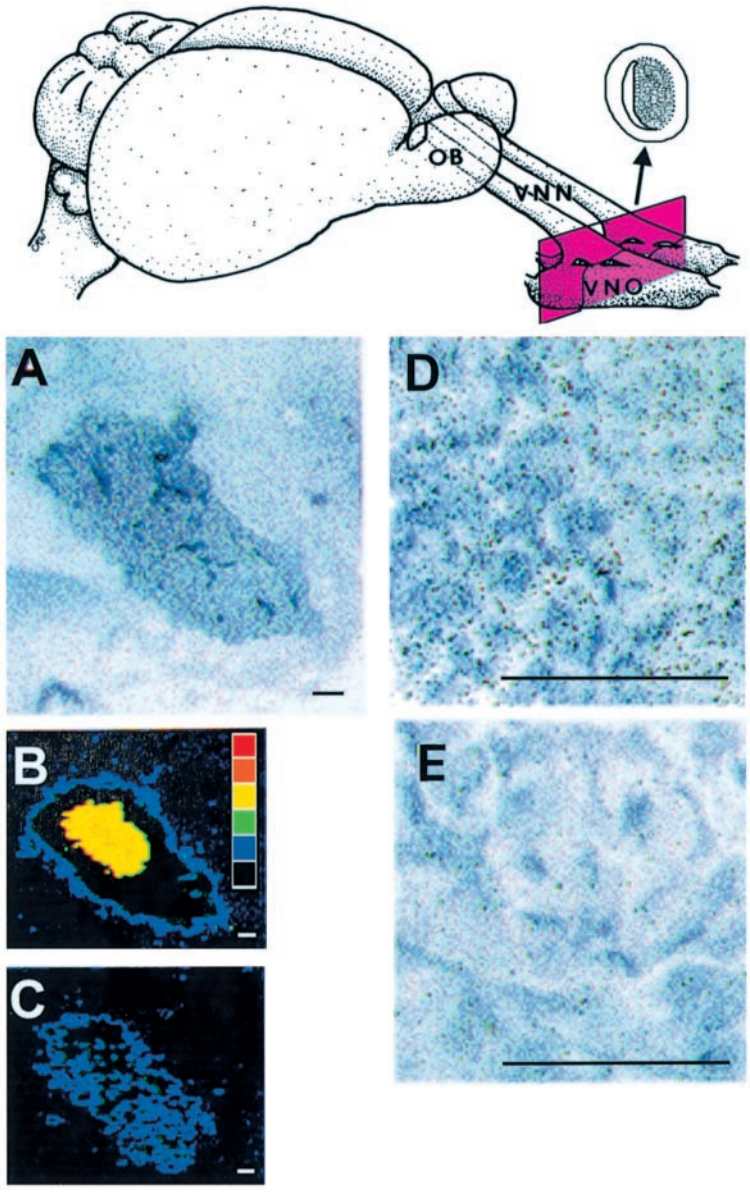

Figure 5 Computer-generated color images and light-field micrographs of [^{125}I]buserelin (GnRH agonist) binding density in coronal sections of adult prairie vole vomeronasal mucosa. **(A)** Histological section of VNO mucosa stained with cresyl violet following fresh-frozen sectioning and post-fixation. **(B)** Computer-generated color image of silver grain density over vomeronasal epithelium incubated in [^{125}I]buserelin (adjacent tissue section to that seen in A). Yellow represents high grain density, whereas blue indicates very low grain density. **(C)** Computer-generated color image of silver grain density over vomeronasal epithelium incubated in [^{125}I]buserelin with excess non-radioactive GnRH (control; adjacent tissue section to that seen in B). **(D)** High magnification light micrograph of vomeronasal sensory epithelium incubated in [^{125}I]buserelin. **(E)** High magnification light micrograph of vomeronasal sensory epithelium incubated in [^{125}I]buserelin with excess of non-radioactive GnRH. Inset: diagram of vole brain and vomeronasal system showing the plane of section (pink rectangle oriented in the coronal plane) of VNO mucosal tissue for receptor binding/autoradiography. Tissue sections in A–C are comparable to the illustration of a vomeronasal mucosal section indicated by the arrow in the inset diagram. OB, olfactory bulb; VNN, vomeronasal nerve, VNO, vomeronasal organ. Bars = 50 μm.

anatomical observations suggest that GnRH acts mainly on the vomeronasal system in this species. A similar tight anatomical association of GnRH-ir neurons with the vomeronasal system has been reported in other rodents such as hamster (Wirsig and Leonard, 1986) as well as in developing rat vomeronasal nerves (Yoshida *et al.*, 1995, 1999). However, it should be noted that GnRH could be secreted locally into the blood stream to travel to other chemosensory regions in the nasal cavity (Jennes, 1986). This is supported by the observation that GnRH modulates olfactory neuron responses to odors (Wirsig-Wiechmann *et al.*, 2000).

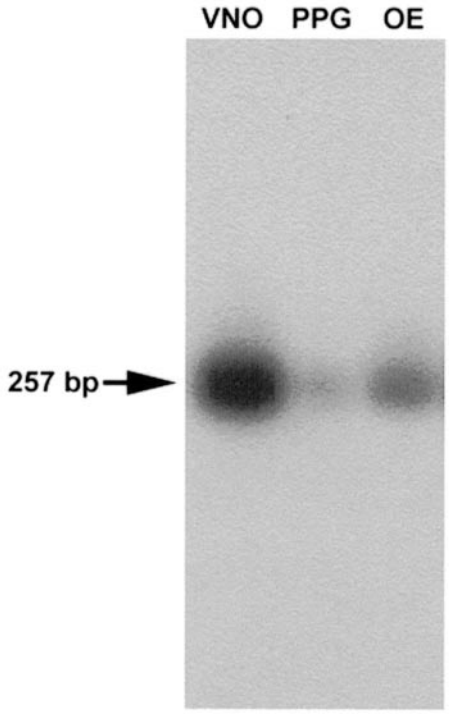

Figure 6 Southern blot of RT–PCR products amplified from adult male vole VNO, pterygopalatine ganglion (PPG) and olfactory epithelium (OE). The PCR products were hybridized with full-length ^{32}P-GnRH receptor cDNA. The labeled GnRH receptor cDNA hybridized specifically to a band of the predicted size (257 bp).

Binding of the GnRH agonist, buserelin, was the highest over the chemosensory vomeronasal neurons. This finding represents the first description of GnRH binding in the chemosensory epithelium of mammals and the second report of GnRH binding in the chemosensory epithelium of vertebrates. Our previous work demonstrated GnRH agonist binding in the olfactory mucosa of tiger salamanders (Wirsig-Wiechmann and Jennes, 1993). Together, these studies suggest that GnRH can influence the processing of chemosensory information, first at the receptor cell level, and that this modulatory role of GnRH in the nasal cavity is common among vertebrates. This hypothesis has been supported by our patch-clamp studies of mudpuppy olfactory mucosa, in which we showed that GnRH modulated voltage-dependent currents (Eisthen *et al*., 2000), and isolated rodent olfactory and vomeronasal receptor cells, in which we showed that GnRH modulated odor-induced currents (Wirsig-Wiechmann *et al*., 2000). In fact, in this latter study in rats, the odor response of one vomeronasal sensory neuron was completely eliminated by GnRH.

A likely natural source of GnRH in the chemosensory mucosa is the nervus terminalis, a GnRH-ir neural plexus in the nasal cavity and rostral forebrain. Our observation of GnRH neural cell bodies and fibers within the vomeronasal epithelium of the neonatal animal may represent a late developmental stage of this system, when neurons are still migrating centrally from the vomeronasal organ. Cell bodies and fibers were not observed in the adult sensory vomeronasal epithelium. The most peripheral immunoreactive fibers in the adult were found within vomeronasal nerves on the nasal septum, just dorsal to the vomeronasal organ. Therefore, in the adult, GnRH would have to diffuse along vomeronasal nerves and through the lamina propria of the vomeronasal organ to reach the sensory epithelium. Another route to the vicinity of the sensory epithelium could be through the microvasculature supplying the lamina propria of the vomeronasal organ. There is an extensive vascular network surrounding the vomeronasal mucosa. We have observed GnRH-ir tangles of fibers surrounding blood vessels just dorsal to the vomeronasal organ, especially in neonatal animals.

Receptors for GnRH are found in many tissues, including the central and peripheral nervous systems. In the brain, GnRH binding sites are abundant in olfactory and limbic areas (Jennes *et al*., 1988), suggesting that there may be intracerebral modulation of olfactory function by GnRH. In support of this, we have observed a dense innervation of the accessory olfactory bulb and entire posterior bulb neck region by GnRH-ir fibers in voles as well as in hamster (Wirsig and Leonard, 1986). This latter region contains 'necklace glomeruli' (Shinoda *et al*., 1989, 1993; Zufall and Munger, 2001) that receive projections from several specialized groups of olfactory receptors (Juilfs *et al*., 1997; Ring *et al*., 1997). The function of the necklace glomeruli throughout an animal's life is not yet known, but there is evidence that it may play a role in pheromone detection during suckling in neonatal rats (Greer *et al*., 1982). The density of GnRH fibers in proximity to these glomeruli suggests to us that they may have a general function related to different aspects of reproduction, perhaps the initial detection of pheromones by the olfactory system and the setting up of odor attractions.

In contrast, in the tiger salamander, GnRH-ir projections are concentrated in medial olfactory nerve projections to rostral regions of the olfactory mucosa and within the olfactory bulb (Wirsig and Getchell, 1986), but are less prevalent in vomeronasal nerves and are rarely seen within the accessory bulb. This species difference in the location of GnRH-ir neurons and fibers may not have significant functional relevance if GnRH accesses the various chemosensory regions via the vasculature. In mammals and most other vertebrates, GnRH accesses the pituitary via the vascular portal system. However, in fish the GnRH fibers project directly into the anterior pituitary (Yamamoto *et al*., 1998). In both cases, GnRH acts to release hormones from the pituitary. Perhaps, the proximity of GnRH-ir fibers to specific areas indicates the regions of acute effects of GnRH, while those areas at greater distance experience the longer-term chronic effects of GnRH. In support of this, the hippocampus has the highest number of GnRH receptors in the brain (Ruebi and Maurer, 1985; Jennes *et al*., 1988, 1990,

1997) and yet contains very few, if any, GnRH fibers (Barry *et al*., 1985).

GnRH is continually released from GnRH neurons along the nervus terminalis by their autonomous activity (Oka, 1992; Abe and Oka, 2000), but this release may increase or decrease depending on hormonal—for example melatonin (Wirsig-Wiechmann, 1993)—and neural inputs (Yamamoto and Ito, 2000). In fact, GnRH itself can be self-regulating; GnRH released from one nervus terminalis neuron can increase the activity and therefore secretion of other GnRH neurons (Abe and Oka, 2000).

The effect of GnRH on peripheral chemosensory cells may involve changes in the concentration of GnRH rather than its presence or absence, as well as changes in the number of GnRH receptors in the target tissue. Electrophysiological recording of mudpuppy olfactory neurons has demonstrated that twice as many olfactory receptor neurons respond to GnRH during the breeding season as at other times. The amount of GnRH released may depend on factors controlling its synthesis. Estrogen has been shown to increase the synthesis of GnRH in brain neurons (Rosie *et al*., 1990) and also appears to increase GnRH synthesis in the nervus terminalis in *Xenopus laevis* as demonstrated by increased immunoreactivity (Wirsig-Wiechmann and Lee, 1999). Conversely, removal of estrogen via ovariectomy leads to a depletion of GnRH in nervus terminalis neurons (Wirsig-Wiechmann and Lee, 1999) due to increases in release (Jennes and Stumpf, 1986).

The GnRH-containing component of the nervus terminalis thus has the potential to modulate chemosensory information, particularly in the vomeronasal system of rodents at multiple peripheral and central sites. This modulation likely occurs in a controlled fashion based on hormonal and neural inputs to the nervus terminalis GnRH system. In future studies we will attempt to determine the molecular mechanisms of GnRH action on chemosensory neurons and the mechanisms that ultimately control the frequency of GnRH release from the nervus terminalis.

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