Silver Lampreys (*Ichthyomyzon unicuspis*)
Lack a Gonadotropin-Releasing Hormone- and FMRFamide-
Immunoreactive Terminal Nerve

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ABSTRACT

The terminal nerve is a ganglioneuron cranial nerve with peripheral processes that enter the nasal cavity and centrally directed processes that enter the forebrain. Members of all classes of gnathostomes have been found to possess a terminal nerve, some components of which demonstrate immunoreactivity to the peptides Phe-Met-Arg-Phe-NH$_2$ (FMRFamide) and gonadotropin-releasing hormone (GnRH). To explore the possibility that lampreys possess a terminal nerve, we examined the distribution of these peptides in the silver lamprey, *Ichthyomyzon unicuspis*, by using antisera to FMRFamide and to four forms of GnRH. We found cells with FMRFamide-like immunoreactivity in the preoptic area and the isthmal gray region of the mesencephalon, and found labeled fibers throughout the preoptic-infundibular region. Occasional labeled fibers were scattered through many regions of the brain, including the optic nerve and olfactory bulb; however, unlike species that possess a terminal nerve, lampreys have no immunoreactive cells or fibers in the olfactory nerve or nasal epithelia. In addition, we observed GnRH-immunoreactive cell bodies in the preoptic area of all animals and in the ventral hypothalamus of one individual. Most of the labeled fibers extended ventrally to the hypothalamus, with other fibers extending throughout the striatum and hypothalamic-neurohypophyseal region. A few fibers in other regions, including the optic nerve, were also labeled; we detected no immunoreactivity in the olfactory bulb, olfactory nerve, or nasal epithelia. The use of different GnRH antisera resulted in remarkably similar patterns of labeling of both cells and fibers. In summary, we did not observe either GnRH or FMRFamide-like immunoreactivity in the olfactory regions that represent the typical path of terminal nerve fibers, nor were we able to locate a terminal nerve ganglion. We conclude that lampreys may lack a terminal nerve, and that the previously described fiber bundle extending from the nasal sac to the ventral forebrain may constitute an extra-bulbar olfactory pathway.

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Early workers described the nervus terminalis, or terminal nerve, as an anterior cranial nerve that extends from the nasal sac to enter the lamina terminalis, giving the nerve its name (Fritsch, 1878; Pinkus, 1894, 1895; Locy, 1905). The ganglia of the terminal nerve are variably located in the region of the nasal sensory epithelia, the olfactory nerve, or the olfactory bulb. Fibers of the terminal nerve typically project through or across the ventromedial aspect of the olfactory bulb to the ventral telencephalon, terminating in the hypothalamic-preoptic area (see review by Demski, 1993). Owing to the lack of agreed-upon criteria for defining the terminal nerve, many researchers appear to assume that any fibers extending between the nasal cavity and hypothalamic-preoptic area are components of the terminal nerve (Hofmann and Meyer, 1991a, 1992, 1995). Other researchers have adopted an implicit definition that includes immunocytochemical criteria. For example, the terminal nerve of most gnathostomes appears to contain a group of fibers that are immunoreactive for gonadotropin-releasing hormone (GnRH; for review, see Muske, 1993). In some taxa, fibers of the terminal nerve also display immunoreactivity to a substance similar to Phe-Met-Arg-Phe-NH$_2$ (FMRFamide), a compound that was originally isolated as a

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molluscan cardioexcitatory peptide (Stell et al., 1984). In recent years, the terminal nerve has come under increasing scrutiny; because of the lack of a clear definition, the identification of the terminal nerve in some taxa has become difficult and complicated (e.g., von Bartheld et al., 1988; Hofmann and Meyer, 1991a; Szabo et al., 1991a; Schober et al., 1994; Hofmann and Meyer, 1995).

Using various definitions and methods, researchers have described the anatomy of the terminal nerve and its ganglia in members of each class of gnathostomes (see Demski and Schwanzel-Fukuda, 1987). For example, Locy (1905) described the terminal nerve in sharks and other elasmobranchs, as have Demski and his colleagues (Demski et al., 1987). Because the teleost terminal nerve extends processes to the retina as well as to the olfactory epithelium, the terminal nerve complex of teleosts is frequently referred to as the "olfactoretinalis system" (Münz et al., 1981), a designation that has led to some confusion (e.g., Szabo et al., 1991a). The anatomy of the teleost terminal nerve/olfactoretinalis system has received much attention (e.g., Sheldon, 1909; Münz et al., 1982; Demski and Northcutt, 1983; Stell et al., 1984; Fujita et al., 1985; von Bartheld and Meyer, 1986a; von Bartheld et al., 1986; Grober et al., 1987; Schreibman and Margolis-Nunno, 1987; Davis et al., 1988; Oka, 1991; Rama Krishna et al., 1992). A terminal nerve has also been described in the non-teleost actinopterygian fishes Amia (Allis, 1897) and Polypterus (von Bartheld and Meyer, 1986b), as well as in lungfish, Propterus (Pinkus, 1894, 1895; von Bartheld and Meyer, 1988; von Bartheld et al., 1988; Schober et al., 1994). Among amphibians, a terminal nerve has been described in several anuran and urodele species (Herrick, 1905; McKibben, 1911; Wirsig and Getchell, 1986; Muske and Moore, 1988; Schmidt et al., 1988; Hofmann and Meyer, 1989; di Meglio et al., 1991; Wirsig-Wiechmann, 1993) and has been investigated in detail in Xenopus (Hofmann and Meyer, 1989b, 1991a,b, 1992). Although data for most reptilian groups are lacking, the terminal nerve has been described in turtle embryos (Johnston, 1913; Larsell, 1919) and in several avian species, including mallard ducks (von Bartheld et al., 1987), chucks (Wirsig-Wiechmann, 1990), and pigeons (Norgren et al., 1992). Finally, the terminal nerve has been described in many mammals, including such diverse species as guinea pigs (Schwanke-Fukuda and Silverman, 1980), bats (Brown, 1980; Oelschläger and Northcutt, 1992), dolphins (Demski et al., 1990), and several primates (Wirsig and Getchell, 1986; Witkin, 1987a,b).

Given that the presence of a terminal nerve appears to be the primitive condition in gnathostomes, it is of considerable interest to determine whether the nerve is present in their sister group, lampreys. To address this issue, however, we need to establish a working definition of the terminal nerve that is consistent with both the classical meaning and the modern usage of the term, and includes the available anatomical and histochemical data. We propose that the designation "terminal nerve" be used to refer to the anterior cranial nerve that projects as a loose group of fibers between the nasal region and the hypothalamic-preoptic region of the forebrain, passing through or over the surface of the olfactory bulb. In some species, cells of the terminal nerve system also project to the retina. The fibers of the terminal nerve project from a ganglion of group of cells located in the nasal region, along the olfactory nerve, or along the surface of the olfactory bulb; these cells may be somewhat dispersed, as has been described in some teleosts and amphibians (Münz and Claas, 1987; Muske and Moore, 1988). Some, but not necessarily all, of these cells and fibers should be immunoreactive for one or more forms of GnRH and may also show immunoreactivity to FMRFamide-like substances or to other compounds. We suggest that the terminal nerve should be distinguished from the extra-bulbar olfactory pathway, a compact bundle of fibers extending from cells in the olfactory epithelium, bypassing the olfactory bulb, to terminate in the basal forebrain. This projection lacks ganglia, and does not display FMRFamide-like or GnRH immunoreactivity (Hofmann and Meyer, 1991a, 1995). It should be noted that Subhedar and Rama Krishna (1988) have described GnRH-immunoreactive cells in the olfactory epithelium of catfish (Clarias batrachus) that may constitute either scattered, peripheral neurons of the terminal nerve, or unusual GnRH-immunoreactive cells of the extrabulbar olfactory

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**Abbreviations**

- AON: anterior octavolateralis nucleus
- ARN: anterior reticular nucleus
- C: cerebellum
- CSG: central spinal gray
- D: dorsal pallium
- DC: dorsal cell
- Di: diencephalon
- DM: dorsal medial neuropil
- DON: dorsal octavolateralis nucleus
- DT: dorsal thalamus
- FR: fasciculus retroflexus
- G: glomeruli of the olfactory bulb
- Hab: habenula
- Hyp: hypothalamus
- IG: isthmal gray
- IN: intermediate octavolateralis nucleus
- LCG: lateral "cerebellar" gray
- LP: lateral pallium
- LpD: lateral pallium, pars dorsalis
- Lpv: lateral pallium, pars ventralis
- M: medial pallium
- Med: medulla oblongata
- MON: medial octavolateralis nucleus
- MN V: trigeminal motor nucleus
- MN VII: facial motor nucleus
- MRN: middle reticular nucleus
- MT: midbrain tegmentum
- n I: olfactory nerve
- n II: optic nerve
- n VII: facial nerve
- NH: neurohypophysis
- nPC: nucleus of the posterior commissure
- OB: olfactory bulb
- OC: optic chiasm
- OT: optic tectum
- PA: preoptic area
- PnT: pretectum
- PT: posterior tubercle
- PVOT: periventricular layer of the optic tectum
- SCO: subcommissural organ
- SMC: somatic motor column
- SN: septal nucleus
- SN V: trigeminal sensory nucleus
- St: striatum
- Tel: telenucleus
- TN: trochlear nucleus
- TS: torus semicircularis
- VT: ventral thalamus
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pathway; thus, given the available data, these cells cannot be easily categorized according to our proposed definitions.

Both the terminal nerve and extrabulbar olfactory pathway are anatomically distinct from the olfactory system, which contains peripheral receptor neurons with axons that terminate in the glomeruli of the main olfactory bulb. Using these anatomical and histochemical criteria, we undertook the present study in an attempt to clarify discrepancies in the literature concerning the possible presence of a terminal nerve in lampreys.

The terminal nerve appears to have its embryological origin in the nasal placode (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989; Murakami et al., 1992; Chiba et al., 1994; Murakami and Araki, 1994; Northcutt and Muske, 1994; Parhar et al., 1994), one of the series of neurogenic placodes that originated with the earliest craniates (Northcutt and Gans, 1983); thus, the developmental precursor cells for the terminal nerve may be present in lampreys. Furthermore, placement of horseradish peroxidase or coab- lysis into the nasal sac labels a group of fibers that pass through the ventromedial olfactory bulb and terminate in several regions, including the hypothalamus and preoptic area in larval Lamperetria (Meyer et al., 1987; von Bartheld et al., 1987; von Bartheld and Meyer, 1988) and in larval and adult Ichthyomyzon (Northcutt and Puzdrowski, 1988). This projection has been interpreted as constituting a terminal nerve, although attempts to label these fibers with antiserum to mammalian GnRH have failed (Meyer et al., 1987). In addition, the distribution of GnRH immunoreactivity has been described in several genera of lampreys, including Entosphenus (Crim et al., 1979), Lamperetria (Crim et al., 1979b), and Petroemyzon (Nozaki et al., 1984; King et al., 1988; Wright et al., 1994; Tobet et al., 1995).

Most of these authors do not explicitly describe the peripheral distribution of immunoreactivity; however, Wright et al. (1994) state that no GnRH immunoreactivity was observed in the olfactory nerves, and Tobet et al. (1995) found that larval Petroemyzon lack GnRH-immunoreactive cells and fibers in the olfactory nerve and epithelium, suggesting that lampreys lack the GnRH immunoreactivity usually associated with the terminal nerve. Nevertheless, many of these investigations arise from the use of only one or a small number of antiseras to different molecular forms of GnRH, leaving open the possibility that peripheral immunoreactivity was not detected because of the characteristics of the particular antiserum used. Ohtomi et al. (1989) describe the distribution of FMRFamide-like immunoreactivity in the brain of Lamperetria, but make no mention of peripheral immunoreactivity, leaving the reader to assume that no immunoreactivity was observed in olfactory regions. Given the available data, it is not possible to determine whether the pathway that has been described in lampreys by von Bartheld and his colleagues (Meyer et al., 1987; von Bartheld et al., 1987; von Bartheld and Meyer, 1988) and by Northcutt and Puzdrowski (1988) constitutes a terminal nerve or an extra-bulbar olfactory pathway.

The present study was designed to assess the distribution of immunoreactivity to diverse molecular forms of GnRH and of FMRFamide-like immunoreactivity, particularly in the peripheral portions of the olfactory system, and to examine the possibility that the fiber bundle that projects from the nasal sac to the hypothalamic-preoptic area in lampreys displays immunohistochemical characteristics similar to those of the terminal nerve in gnathostomes. Specifically, we used three different antiseras to a form of GnRH that has been isolated from lampreys (lamprey GnRH I, Sherwood et al., 1986), and an antiserum to chicken GnRH II, a form of GnRH that is present in the brains of members of all classes of gnathostomes (King and Millar, 1992). We also used antiseras to forms of GnRH that were originally isolated from salmon and from mammals. In addition, we used an antiserum to FMRFamide. Although FMRFamide was originally isolated from molluscs, the use of such antiseras results in labeling of FMRFamide-like compounds in a wide range of craniates (e.g., Jirikowski et al., 1984; Stell et al., 1984; Wirsig-Wiechmann and Basinger, 1988; Ohtomi et al., 1989; Wirsig-Wiechmann, 1990; Chiba et al., 1991). Three peptides that share the C-terminal sequence Arg-Asp-NH2 have been isolated from the central nervous system of amniotes (chickens, Dockray et al., 1983; cows, Yang et al., 1985); presumably, the antiserum to FMRFamide that produce labeling in the brains of other craniates are recognizing this C-terminal portion of one or several FMRFamide-like peptides that are present.

Using these techniques, we documented the absence of FMRFamide-like immunoreactivity, and of immunoreactivity to all four molecular forms of GnRH, in the olfactory bulb and nasal periphery of adult silver lampreys (Ichthyomyzon unicuspis). We also failed to locate a candidate terminal nerve ganglion. On the basis of our proposed definition, we conclude that the previously described fibers that project from the nasal sac to the hypothalamus and preoptic area in lampreys represent an extra-bulbar olfactory pathway rather than a terminal nerve.

MATERIALS AND METHODS

The subjects for this study were nine healthy adult silver lampreys (Ichthyomyzon unicuspis) of both sexes, obtained from Mason Aquatics (Guttenberg, IA). Lampreys were anesthetized by immersion in tricaine methanesulphonate (MS 222), perfused transcardially with cold 0.1 M phosphate buffer (pH 7.4), followed by cold 4% paraformaldehyde in phosphate buffer. The neurocranium, including the brain and nasal sac, was removed and postfixed for 1 hour at 4°C, then cryoprotected overnight in phosphate buffer containing 20% sucrose. The tissue was embedded in gelatin and fixed overnight in 4% paraformaldehyde-20% sucrose in phosphate buffer. The gelatin block was then frozen and transverse sections, 40 µm thick, were cut on a sliding microtome and collected into cold phosphate buffer with 0.001% sodium azide added. Sections were stored at 4°C until processing. Some sections were treated with 6% hydrogen peroxide in phosphate buffer at 4°C for 5 days and rinsed thoroughly before exposure to the primary antiserum to remove pigment in the tissue underlying the olfactory epithelium.

Immunocytochemical processing was carried out on free-floating sections according to a previously described peroxidase-antiperoxidase (PAP) protocol (Sternberger, 1979; modified as described by Wicht and Northcutt, 1992a). Briefly, sections were washed in phosphate buffer and incubated in primary antiserum for 48-72 hours at 4°C. After another washing, sections were incubated in a secondary antibody, a 1:100 dilution of goat anti-rabbit IgG, for 60 minutes at room temperature. Sections were then washed and incubated in rabbit PAP, diluted 1:300, for 60 minutes at room temperature. After washing, sections were treated with hydrogen peroxide in a solution of nickel and diamino benzidine in 0.1 M acetate buffer (pH 6.0). Sections were later

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washed, mounted, dehydrated, and coverslipped; some series were counterstained with neutral red to facilitate identification of cell groups. In total, sections from six animals were used to determine distribution of GnRH immunoreactivity, and sections from five animals were exposed to anti-FMRFamide.

Seven primary antisera were used: anti-salmon GnRH (PBL-L49, courtesy of Joan Vaughan and Wylie Vale), diluted 1:4,000 in phosphate buffer with 0.3% Triton-X and 0.001% sodium azide added; anti-mammalian GnRH (71227, Incstar), diluted 1:4,000; anti-chicken GnRH II (100, courtesy of Jim Millam), diluted 1:500 to 1:1,000; anti-lamprey GnRH I (21–134, courtesy of Stacia Sower), diluted 1:1,000 to 1:2,000; anti-lamprey GnRH I (1459, courtesy of Judy King), diluted 1:500 to 1:1,000; anti-lamprey GnRH I (1467, courtesy of Judy King), diluted 1:500; and anti-FMRFamide (79127, Incstar), diluted 1:4,000. Two of the antisera to lamprey GnRH I (21–134 and 1467) have been shown to recognize both lamprey GnRH I and lamprey GnRH III (Tobet et al., 1995); the specificity of antisera 1459 is uncertain.

Several different control procedures were employed. In one experiment, primary antiserum was omitted from the procedure. In another, anti-mammalian GnRH was blocked by preabsorption with approximately 85 μM mammalian GnRH (Sigma, L-7154) for 24 hours at 4°C before the tissue was added. Anti-FMRFamide was preabsorbed with 10 μM FMRFamide (Sigma, P-6535). No labeling was observed after any of these control procedures.

Measurements of cell size and fiber diameter were made by using an eyepiece micrometer on a Nikon Labophot microscope. Note that some shrinkage due to fixation and tissue processing may have occurred; thus reported measurements may underestimate actual size but do provide a guide to the relative sizes of various structures.

The original research reported in this paper was conducted according to animal care and use guidelines established by the Society for Neuroscience and the University of California, San Diego.

RESULTS

The different anti-GnRH antisera produced similar patterns of labeling of both cells and fibers, although the resulting label was most clear and intense with the anti-chicken GnRH II antisera. We will therefore describe a general pattern of anti-GnRH immunoreactivity, with exceptions noted. Because the antiserum to GnRH and to FMRFamide labeled different structures, we will describe the distribution of anti-FMRFamide immunoreactivity separately.

Figure 1 depicts a dorsal view of the brain of an adult silver lamprey and indicates levels of the transverse sections charted in Figures 2 and 4.

Distribution of GnRH immunoreactivity

Camera lucida drawings of the distribution of chicken GnRH II immunoreactivity in one individual are shown in Figure 2, and photomicrographs of the GnRH-labeled cell groups in the same individual are shown in Figure 3.

A cluster of cells in the preoptic area was immunoreactive to all forms of GnRH tested in each individual examined. Not all cells in the preoptic area were labeled. We did not observe any labeled cells in the ventral portion of the preoptic area, and only a few scattered cells were labeled in the dorsal portion of the rostral preoptic area (see Figs. 2A, 3A). Cells in the central region of the preoptic area were densely and uniformly labeled with antisera to GnRH. In all animals, the cells in this central preoptic region were arranged in two laminae that ran parallel to the ventricular wall. The innermost lamina consisted of one row of cells, whereas the more external lamina was one to three cells deep. These laminae were separated by a zone that was roughly the width of one cell body. In the rostral portion of the preoptic area, the cell bodies of the innermost lamina were located adjacent to the ventricle, whereas further caudally the innermost lamina of cells was located as much as 30 μm from the ventricle, as can be seen in Figure 3C. In addition, in one animal a small group of labeled cells was found in the neuropil lateral to the preoptic area; this cluster of cells was approximately 100 μm in its rostral-caudal extension and was labeled by all three antisera to lamprey GnRH I.

The GnRH-immunoreactive cells of the preoptic area were bipolar and slightly elliptical in shape, approximately 10–15 μm in diameter. The labeled cells were not morphologically distinguishable from the unlabeled cells in the same region that were stained with neutral red. Within the central portion of the preoptic area, the cells possessed a short, heavily staining, thick process that extended to the ventricular wall or up to 1 μm into the ventricle. These processes terminated in densely staining, club-shaped thickenings. This ventricular process was approximately 2 μm in diameter, and the terminal thickening was approximately 3 μm in diameter in our material. A slightly thickened process (approximately 1 μm diameter) arose from the opposite pole of the cell; although these fibers appeared not to branch, dense labeling in this region precluded the possibility of definitive observations. The somata of labeled cells in the dorostral portion of the preoptic area resembled those in the more central region, although the former lacked thickened processes and did not appear to contact the ventricle (Fig. 3A).

In one animal, a male that was examined early in the spawning season, two clusters of cells within the ventral hypothalamus were immunoreactive to anti-salmon or anti-chicken GnRH II but not to the antisera to mammalian GnRH. Anti-salmon GnRH labeled some of the cells in the rostral portion of the ventral hypothalamus (Fig. 3C), and approximately ten cells in the caudal portion of the ventral hypothalamus were lightly labeled with both anti-salmon and anti-chicken GnRH II (Fig. 2D). The rostral and caudal cell groups were separated by at least 200 μm, and the morphology of the labeled cells differed between the two groups. The labeled cell bodies in the rostral portion of the ventral hypothalamus were oval-shaped and bipolar, with a diameter of 6–8 μm across the longest dimension of the soma. The ventricular process of these cells was thickened (approximately 1 μm diameter) and terminated in a heavily staining knob that was 2–3 μm in diameter. The process extending from the other pole of the cell body was not thickened. Overall, these cells resembled the GnRH-immunoreactive cells of the preoptic area. In contrast, the labeled cells in the caudal portion of the ventral hypothalamus were quite round and had large somata that ranged from 8 to 10 μm in diameter; the processes of these cells
could not be distinguished. These labeled cells were not morphologically different from adjacent cells that were counterstained with neutral red.

In all animals examined, the GnRH-immunoreactive fibers tended to form scattered and diffuse projections, as can be seen in Figure 3C, rather than forming discrete bundles or pathways (Fig. 2). The thick fibers radiating outward from the preoptic cell group extended dorsally through the striatum, laterally to the lateral pallium, ventrally throughout the hypothalamic-infundibular region (Fig. 2A), rostrally to the olfactory bulb (Fig. 2A), and caudally to approximately the boundary between the telencephalon and diencephalon (Fig. 2C, D). The terminal fields of these thickened fibers could not be determined. A dense projection of fine fibers extended into and throughout the neurohypophysis, and scattered fine fibers were also seen extending into the medial pallium, striatum, lateral pallium, optic nerve, dorsal pallium, dorsal thalamus, posterior tubercle, pretectum, tegmentum, and torus semicircularis. Occasional fine fibers were observed extending through the mid- and hindbrain as far caudally as the spinal cord (Fig. 2I). These fine fibers appeared to follow a rostrocaudal course, as only short segments of fibers were visible in our transverse sections. The distribution and morphology of GnRH-immunoreactive fibers were similar in the individual in which immunoreactive cells were found in the ventral hypothalamus. No GnRH-immunoreactive fibers were seen in the nasal epithelia, olfactory nerve, or olfactory bulb in any individual.

As illustrated in Figure 3C, most of the GnRH-immunoreactive fibers had a conspicuously beaded appearance, suggesting the possible presence of presynaptic portions of synapses en passant. These varicosities were of a similar size and distribution on both the thin and thickened fibers extending into and throughout the neuropil, although they were not present on the short, thick processes extending to the ventricle from the central preoptic and ventral hypothalamus cells.

**Distribution of FMRFamide-like immunoreactivity**

The distribution of labeled cells and fibers that display FMRFamide-like immunoreactivity in one individual is shown in camera lucida drawings in Figure 4, and photomicrographs of labeled cell groups from this and another individual are shown in Figure 5.

In all individuals examined, antiserum to FMRFamide labeled cells in the dorsal hypothalamus, generally in a
cluster at least 10 μm from the ventricle (Figs. 4C,D, 5A). In the more rostral portion of the dorsal hypothalamus the labeled cells formed a tight group, whereas in the caudal portion the labeled cells were interspersed among unlabeled cells. Throughout the dorsal hypothalamus, the labeled cells were slightly elliptical bipolar cells measuring 7–12 μm wide and × μm long.
Fig. 3. Photomicrographs of gonadotropin-releasing hormone (GnRH)-immunoreactive cells and fibers in transverse sections through the brain. This adult silver lamprey is the same individual used for the camera lucida drawings in Figure 2. A: Cells and fibers in the rostral preoptic area, labeled with anti-chicken GnRH II. The level of this photograph corresponds to that illustrated in Figure 2A. B: Cells and fibers in the caudal preoptic area, labeled with anti-chicken GnRH II. Note that the fibers in the region of the labeled cells are thicker than those found more distally, as in the ventral (bottom) portion of this photograph. This photograph is taken from the section drawn in Figure 2B. C: Cells in the ventral hypothalamus showing anti-salmon GnRH immunoreactivity. Labeled cell bodies are clustered along the ventricle, which is to the right of the section. Note the beaded appearance of the fibers. Of the six individuals examined with anti-GnRH antisera, ventral hypothalamic cells were labeled only in this one individual. This section lies just rostral to the section illustrated in Figure 2B. Dorsal is toward the top of each micrograph. Scale bars = 100 μm.
μm across the long axis. Each cell extended toward the ventricle a short, thick, heavily labeled process that was 2–3 μm in diameter; many of these processes protruded into the ventricle, as shown in Figure 5A. At the opposite pole, each cell also possessed a thick process extending into the surrounding neuropil. These processes were 2–3 μm in diameter near the soma but tapered rapidly, and within roughly 200 μm they were the same diameter as other labeled fibers throughout the brain.

In two individuals, a group of cells in the mesencephalon was lightly labeled. These cells formed an arc extending throughout the isthmal gray region and rostral portion of the trigeminal sensory nucleus, ventral to the so-called cerebellar region (Figs. 4F, 5B). Only a fraction of the somata in this region was labeled. The cells that showed FMRFamide-like immunoreactivity were 7–10 μm in diameter and were multipolar, with fine, branching processes extending in all directions.

Fibers that were immunoreactive for FMRFamide-like compounds did not form discrete bundles, but were scattered diffusely throughout diverse regions of the brain. Furthermore, most fibers appeared to pass in the rostro-caudal plane, as indicated by the short segments of fibers visible in our transverse sections; thus, the paths followed...
by groups of fibers could not be followed with certainty. We observed labeled fibers in many telencephalic structures, including the olfactory bulbs, lateral pallium, preoptic area, optic nerves, and hypothalamus, as well as in the neurohypophysis and many portions of the diencephalon, midbrain, hindbrain, and into the spinal cord (see Fig. 4). Despite the extensive distribution of fibers with FMRFamide-like immunoreactivity throughout the brain, no labeled fibers were found in or near the olfactory nerves or olfactory epithelium of any of the individuals examined.

In general, fibers showing FMRFamide-like immunoreactivity contained bead-like varicosities. Interestingly, the thickened portions of the fibers in the region of the dorsal hypothalamus were not beaded. The varicosities on the
fibers of cells with FMRFamide-like immunoreactivity were spaced more widely along the course of a fiber than was seen with GnRH-immunoreactive fibers. The distribution and morphology of labeled cells and fibers did not appear to vary according to the sex or reproductive condition of the individuals examined. We did not observe any cells that displayed both GnRH and FMRFamide-like immunoreactivity.

DISCUSSION

Distribution of FMRFamide-like and GnRH immunoreactivity in lampreys

The distribution of FMRFamide-like and GnRH immunoreactivity that we observed in the brain of adult silver lampreys corresponds closely to patterns described using similar antisera with adult lampreys of other species.

Two groups of cells were found to contain FMRFamide-like material. A large group of cells in the dorsal hypothalamus was labeled with antisera to FMRFamide; these cells correspond to the “rostral,” “dorsal,” and “caudal” cell groups that Ohnoki and colleagues (1989) described in Lampetra japonica. The labeled cells of the dorsal hypothalamus were bipolar and extended a short process to the edge of, or into, the infundibular recess and third ventricle (Fig. 5A). Morphologically similar processes can be seen in Lampetra (Ohnoki et al., 1989). Presumably these cells release a FMRFamide-like peptide into the cerebrospinal fluid, although these fibers may also constitute dendritic processes. In addition, in two of the five individuals exposed to the anti-FMRFamide antisera, we observed a scattered group of labeled cells in the midbrain, near the sensory nucleus of the trigeminal nerve. This cluster of cells has not been described in lampreys. These fusiform, multipolar cells were fairly lightly labeled, indicating that the midbrain cells contain either a lower concentration of immunoreactive peptide or contain a different peptide with lower affinity for the anti-FMRFamide antisera than do the cells of the dorsal hypothalamus.

Large, bipolar GnRH-immunoreactive cells have been described in the preoptic area of adult Entosphenus triden-tata (Crim et al., 1979a), Lampetra richardsoni (Crim et al., 1979b), and both larval and adult Petromyzon marinus (King et al., 1988; Wright et al., 1994; Tobet et al., 1995) and were labeled in our Ichthyomyzon material with antisera to four forms of GnRH. Furthermore, we found two laminae of immunoreactive cells in the preoptic area; this configuration was first observed by Crim et al. (1979a), who demonstrated the presence of a row of aldehyde fuchsin-positive cells juxtaposed between these laminae in Entosphenus. In our material, the distance between the two immunoreactive laminae is approximately the width of one soma, possibly indicating the presence of a similar row of cells. The ventricular processes of the immunoreactive cells terminate in club-like swellings that extend to the edge of, and in many cases protrude into, the ventricle, as has been reported for other lamprey species (Crim et al., 1979a; Crim, 1981; King et al., 1988; Tobet et al., 1995). The dense staining of these processes indicates that they contain GnRH, which may be released into the ventricular system as a method of transporting GnRH to the anterior pituitary, as lampreys lack an anatomical connection between the hypothalamus and adenohypophysis (Gorbman, 1965; King et al., 1988).

The GnRH-immunoreactive fibers that were observed throughout the rest of the brain contained closely spaced varicosities, as described in Entosphenus and Lampetra by Crim and his colleagues (1979a,b). Thin fibers displaying FMRFamide-like immunoreactivity contained similar varicosities that occurred at greater intervals along the length of the fibers. The majority of thin fibers displaying GnRH or FMRFamide-like immunoreactivity appeared to pass in a rostral-caudal direction (King et al., 1988; Ohnoki et al., 1989), obscuring their terminal fields.

We obtained the darkest staining and lightest background in the cells of the preoptic area and in all labeled fibers with the antisera directed against chicken GnRH II. Similar but lighter labeling was obtained with the antisera to salmon and lamprey GnRH I, and the antisera against mammalian GnRH produced light labeling and resulted in higher background staining than was observed with the other antisera. Control experiments indicate a high specificity of staining. The similar distribution of GnRH immunoreactivity observed with antisera to four different molecular forms of GnRH indicates a high degree of cross-reactivity between our antisera and the form(s) of GnRH present in the cells of the preoptic area and in the fibers throughout the brain. Indeed, two of the antisera to lamprey GnRH I that we used have been shown to cross-react with lamprey GnRH III (Tobet et al., 1995).

In addition to the GnRH-immunoreactive cell group in the preoptic area, we observed two groups of labeled cells in the ventral hypothalamus of a single adult male that was examined early in the spawning season. These cell groups were both labeled by the anti-salmon GnRH antisemur, and we observed somewhat lighter label in the caudal group with the antisemur to chicken GnRH II. We found no label in these cell groups in sections reacted with antisemur to mammalian GnRH. Given this pattern of affinity for the different antisera, it is possible that these hypothalamic cell groups contained a different molecular form of GnRH than that which is present in the preoptic cell group. It is important to note that because a control blocking experiment was not conducted with this individual, the possibility of nonspecific labeling cannot be excluded. Nevertheless, Tobet et al. (1995) present strikingly similar data from larval Petromyzon, in which two groups of cells in the ventral hypothalamus were only lightly labeled with antisemur to lamprey GnRH I and III. During metamorphosis, the cells of the more caudal group became elongated and showed greater immunoreactivity to lamprey GnRH III. Perhaps, as suggested by Tobet et al. (1995), these cell groups are most prominent or active at metamorphosis, and the single animal in which we detected these cell groups was less mature than the other individuals examined. Similarly, the expression of GnRH in the ventral hypothalamus of the teleost Haplochromis burtoni has been shown to depend on the individual’s size and reproductive status (White and Fernand, 1993; Francis et al., 1994).

It is interesting to note that we did not find GnRH immunoreactivity in several regions that typically contain GnRH in gnathostomes. For example, lampreys appear to lack the group of chicken GnRH II-containing cells that is found in the posterior diencephalon or mesencephalon of most gnathostomes (Muske, 1993), although lampreys may possess a cell group containing a unique form of GnRH that was undetectable using our antisera. In addition, we failed to find GnRH immunoreactivity in or near the nasal sac, olfactory nerve, or olfactory bulb, the typical locations of
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GnRH immunoreactivity in species that possess a terminal nerve. Recent evidence indicates that the GnRH-immunoreactive cells in the ventral forebrain of at least some gnathostomes originate in the nasal placode and migrate along the terminal nerve into the forebrain, whereas the immunoreactive cells of the midbrain have a different embryonic origin (Murakami and Arai, 1994; Chiba et al., 1994; Murakami and Arai, 1994; Northcutt and Muske, 1994; Parhar et al., 1994). Given that lampreys may lack a GnRH-containing midbrain cell group, and lack a terminal nerve (as we will argue below), the development and anatomical organization of the GnRH system of lampreys appears to be somewhat different than that of gnathostomes. Based on the presumed dissimilarity of embryonic origin, Muske (1993) has suggested that the GnRH-immunoreactive cell group of the preoptic area in lampreys is not homologous with the immunoreactive cell group in the ventral forebrain of gnathostomes. Nevertheless, similarity of embryological origin is not a strong criterion for homology (de Beer, 1971), and an outgroup analysis of effenter and afferent connectivity is required to establish the evolutionary relationships among these cell groups (Striedter and Northcutt, 1991). Hagfish constitute the sister group to lampreys and gnathostomes, and recent data indicate that the Pacific hagfish, Eptatretus stouti, possesses a GnRH-immunoreactive cell group in the preoptic area, and that fibers of these cells project to the neurohypophysis (Braun et al., 1995). A similar projection exists in lampreys, as indicated by our observations and by those of King et al. (1988), and a ventral forebrain group of GnRH-immunoreactive cells projecting to the hypothalamus has been described in all gnathostomes examined to date (reviewed in Muske, 1993). In contrast, the presence of ventricular processes appears to be unique to the GnRH-immunoreactive cells of hagfish and lampreys. Thus, the data are equivocal. It appears possible that the presence of a GnRH-immunoreactive cell group in the preoptic-hypothalamic area is a primitive feature of craniate nervous systems (Braun et al., 1995), although differences in embryological origin and ventricular contacts call into question the homology of this cell group across craniates.

Finally, occasional fibers in the optic nerve were immunoreactive with antisera to GnRH or to FMRFamide. It is tempting to speculate that these fibers are homologous with the retinopetal component of the terminal nerve system that has been described in many teleosts and in land frogs (e.g., Münz et al., 1982; Wirsig-Wiethmann and Basinger, 1988). Unfortunately, we do not yet have sufficient data regarding the organization of retinal projections in lampreys to determine the relation between the fibers labeled in our material and those described in other vertebrates.

Do lampreys possess a terminal nerve?

We did not observe GnRH or FMRFamide-like immunoreactivity in fibers of the nasal sac or olfactory nerve, and we did not find immunoreactive cells in the nasal sac, along the course of the olfactory nerve, or in the olfactory bulb. Other researchers examining the distribution of GnRH or FMRFamide-like immunoreactivity in the brains of lampreys have not observed labeled cells or fibers in these regions (Crim et al., 1979a,b; King et al., 1988; Ohtomi et al., 1989). Using a counterstain (neutral red), we were unable to identify a group of cells in the nasal sac, along the olfactory nerve, or in the olfactory bulb that might constitute a ganglion of the terminal nerve, although we may have failed to detect a small group of dispersed ganglion cells. These data are consistent with the interpretation that lampreys lack a terminal nerve and its ganglion.

In contrast, previous researchers have described a group of fibers labeled by application of horseradish peroxidase or cobalt lysine into the nasal sac of larval or adult lampreys (Ichthyomyzon, Northcutt and Puzdzrowski, 1988; Lampera, Meyer et al., 1987; von Bartheld et al., 1987; von Bartheld and Meyer, 1988). These fibers project from the nasal sac through the olfactory bulb to the ventral forebrain, a path similar to that of the terminal nerve in gnathostomes. Nevertheless, we did not observe immunoreactive fibers along this pathway in the forebrain, and Meyer et al. (1987) report that attempts to label this pathway with antiserum to mammalian GnRH failed. Furthermore, Meyer et al. (1987) describe a putative terminal nerve ganglion at the anterior margin of the olfactory bulb in larval Lampera that was retrogradely labeled by placement of horseradish peroxidase into the nasal sac; however, these authors do not mention this cell group in later descriptions of the terminal nerve system of lampreys (von Bartheld et al., 1987; von Bartheld and Meyer, 1988). Using similar techniques, Northcutt and Puzdzrowski (1988) failed to locate a ganglion in either juvenile or adult Ichthyomyzon. Although we do not consider Meyer et al.’s (1987) description as constituting strong evidence for the existence of a terminal nerve ganglion, the possibility cannot be ruled out. Taken together, these findings appear to be contradictory, and it is not obvious whether lampreys possess or lack a terminal nerve. Several hypotheses can account for these data. 1) The terminal nerve may be present in larval lampreys, but may regress at metamorphosis such that it is absent in adults. 2) Lampreys may possess a terminal nerve throughout the life cycle, but our antisera may have failed to detect the GnRH and/or FMRFamide-like compounds that are present. 3) Lampreys may possess a terminal nerve that lacks GnRH and FMRFamide-like compounds. 4) Lampreys may lack a terminal nerve. We will treat these in order.

The terminal nerve may be present in larval lampreys, but may regress at metamorphosis such that it is absent in adults. A putative terminal nerve ganglion has been described only in larval lampreys (Meyer et al., 1987), and may be lost at metamorphosis such that it is absent in adults. As noted above, however, such a ganglion has not been described in subsequent studies of larval lampreys (von Bartheld et al., 1987; von Bartheld and Meyer, 1988). Furthermore, this hypothesis does not account for the lack of GnRH immunoreactivity in the olfactory nerve and epithelium of larval lampreys (Crim et al., 1979b; Meyer et al., 1987; Wright et al., 1994; Tobet et al., 1995). Taken alone, this hypothesis does not account for the available data.

Lampreys may possess a terminal nerve throughout the life cycle, but our antisera may have failed to detect the GnRH and/or FMRFamide-like compounds that are present. It is possible that the terminal nerve of lampreys contains a FMRFamide-like peptide that is sufficiently different from those present in gnathostomes that it cannot be detected with our antiserum. In addition, although we used three antisera to one form of lamprey GnRH (lamprey GnRH I), the terminal nerve may contain one of the other two molecular forms of GnRH that have been isolated from lampreys (Sherwood et al., 1986; Sower et al., 1993), or it may contain a different form of GnRH. Nevertheless, the
antiserum to mammalian GnRH that we used almost certainly detects the N- or C-terminal portions of the GnRH molecule (King et al., 1988). These portions have been highly conserved across all forms of GnRH that have been sequenced to date (Sherwood et al., 1993) and therefore would be expected to be labeled if GnRH were present. Furthermore, each of our antisera labeled cells and fibers in other portions of the nervous system. We consider unlikely the possibility that the terminal nerve contains unique forms of GnRH and FMRFamide-like compounds that cannot be detected with our antisera, but we cannot rule it out.

**Lampros may possess a terminal nerve that lacks GnRH and FMRFamide-like compounds.** Von Bartheld and his colleagues have suggested that lampros possess a terminal nerve that lacks GnRH (Meyer et al., 1987; von Bartheld et al., 1987; von Bartheld and Meyer, 1988). Similarly, recent data indicate that lizards either lack a terminal nerve, or possess a terminal nerve that lacks a GnRH-immunoreactive component (Masucci et al., 1992; D'Aniello et al., 1994). In addition, the terminal nerve of some urodele amphibians lacks FMRFamide-like immunoreactivity (Muske and Moore, 1988), as does that of mammals (Muske, 1993). We cannot rule out the possibility that the terminal nerve of lampros may lack both GnRH and the FMRFamide-like substance(s) present in that of many gnathostomes.

**Lampros may lack a terminal nerve.** The fibers that have previously been thought to constitute the terminal nerve (von Bartheld et al., 1987; Northcutt and Puzdrowski, 1988; von Bartheld and Meyer, 1988) may represent an extra-bulbar olfactory pathway (EBP, Szabo et al., 1991b). Because the EBP has only recently been recognized, previous studies of the terminal nerve may have confounded the identity of the two pathways, as appears to have occurred with classical studies of the terminal nerve in frogs and urodèles (Herrick, 1909; McKibben, 1911; discussed in Hofmann and Meyer, 1991a). Furthermore, the phylogenetic distribution of the EBP has not yet been determined. The most thorough studies of the anatomy of the EBP have been conducted with the frog *Xenopus laevis* (Hofmann and Meyer, 1991a,b, 1992) and other amphibians (Hofmann and Meyer, 1989a). The EBP has also been described in a large number of actinopterygian fishes, including salmonids (Bazer et al., 1987; Riddle and Oakley, 1992; Becerra et al., 1994) and other teleosts (gasterosteiforms, Honkanen and Ekström, 1990; gymnotiforms, Szabo et al., 1991b; perciforms, Hofmann and Meyer, 1995), as well as *Amia, Acipenser*, and two polypteroform species (Hofmann and Meyer, 1995). A similar projection has been described in adult rats (Monti Graziaidei, 1993).

In amphibians and actinopterygians, the EBP comprises a group of fibers that project from receptor-like cells in the olfactory epithelium to the ventral forebrain, with some fibers projecting to more caudal regions. Although these fibers pass along the olfactory nerve and through the olfactory bulb, they do not terminate in the glomeruli of the olfactory bulb, as do the axons of other receptor cells in the olfactory epithelium. The tract of the EBP is more compact, and follows a different pathway than do the fibers of the terminal nerve (Hofmann and Meyer, 1991a, 1992). Furthermore, the fibers of the EBP can be labeled with soybean agglutinin, which does not bind to fibers of the terminal nerve (Hofmann and Meyer, 1991b, 1995), and Monti Graziaidei (1993) has demonstrated that the fibers of the EOB in rats express a protein specific to the olfactory system, called olfactory marker protein, which is not found in any portion of the terminal nerve. The degree to which these findings can be generalized to other taxa remains to be determined.

Both the terminal nerve and EBP can be labeled by injection of horseradish peroxidase into the nasal cavity (Hofmann and Meyer, 1989a); thus, the terminal nerve and EBP can best be discriminated by the presence of a ganglion, which is unique to the terminal nerve, by their different projection pathways, and by the immunocytochemical and histochemical criteria discussed above. Because the fiber pathway that previously has been thought to constitute the lamprey terminal nerve lacks a ganglion, lacks GnRH and FMRFamide-like substances, and forms a compact bundle through the telencephalon, this pathway more closely resembles an EBP than a terminal nerve. This hypothesis must be tested directly, by examination of the peripheral projections of these fibers and of their soybean agglutinin-binding properties, before the identity of these fibers can be resolved.

Nevertheless, we suggest that lampros may not possess a terminal nerve, as they appear to lack an appropriate ganglion and do not display GnRH or FMRFamide-like immunoreactivity in the peripheral regions associated with the olfactory system. Hagfish also appear to lack a terminal nerve, as indicated by morphological studies, as well as by examinations of the distribution of the GnRH and FMRFamide-like immunoreactivity in the brain of various *Eptatretus* species (Crim et al., 1979a; Jirikowski et al., 1984; Wicht and Northcutt, 1992a,b; Braun et al., 1995). Given that the terminal nerve is present in all classes of gnathostomes, but may be absent in lampros and hagfish, we conclude that the terminal nerve may be an evolutionary innovation in gnathostomes.

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